A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E

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In the yeast *Saccharomyces cerevisiae* a small protein named p20 is found associated with translation initiation factor eIF4E, the mRNA cap-binding protein. We demonstrate here that p20 is a repressor of cap-dependent translation initiation. p20 shows amino acid sequence homology to a region of eIF4G, the large subunit of the cap-binding protein complex eIF4F, which carries the binding site for eIF4E. Both, eIF4G and p20 bind to eIF4E and compete with each other for binding to eIF4E. The eIF4E–p20 complex can bind to the cap structure and inhibit cap-dependent but not cap-independent translation initiation: the translation of a mRNA with the 67 nucleotide Ω sequence of tobacco mosaic virus in its 5' untranslated region (which was previously shown to render translation cap-independent) is not inhibited by p20. Whereas the translation of the same mRNA lacking the Ω sequence is strongly inhibited by p20. Disruption of *CAF20*, the gene encoding p20, stimulates the growth of yeast cells, overexpression of p20 causes slower growth of yeast cells. These results show that p20 is a regulator of eIF4E activity which represses cap-dependent initiation of translation by interfering with the interaction of eIF4E with eIF4G, e.g. the formation of the eIF4F-complex.

Keywords: eIF4E/eIF4G/4E-BPs/*Saccharomyces cerevisiae*/translation initiation

Introduction

Most eukaryotic mRNAs bear a 'cap' structure m⁷GpppX (where X is any nucleotide and m is a methyl group) at their 5' terminus (Shatkin, 1985). This structure is recognized by the cap-binding protein eIF4E (Sonenberg *et al.*, 1978). This 24 kDa polypeptide is a subunit of the cap-binding complex eIF4F (Tahara *et al.*, 1981; Edery *et al.*, 1983; Grifo *et al.*, 1983) which, in higher eukaryotes, also contains eIF4A and eIF4G/p220 (Sonenberg, 1988). Together with eIF4B, eIF4F catalyzes the unwinding of RNA secondary structure in the 5' UTR (untranslated region) of mRNA (Rozen *et al.*, 1990) and the binding of the 40S ribosomal subunit•met-tRNA•eIF2•GTP complex (43S-preinitiation complex) to the mRNA (Merrick and Hershey, 1996; Pain, 1996).

Regulation of cap-binding activity is essential for normal

growth and proliferation of mammalian cells. Increasing the expression of eIF4E in rodent fibroblasts, either by cDNA transfection (Lazaris-Karatzas *et al.*, 1990) or by microinjecting the protein (Smith *et al.*, 1990) stimulates cell growth and causes the cells to acquire a transformed phenotype. The transforming activity of high levels of eIF4E in mammalian cells may be explained by increased eIF4F activity stimulating the translation of mRNAs for growth promoting genes such as cyclin D1 (Rosenwald *et al.*, 1993), ornithine decarboxylase (Shantz and Pegg, 1994) and c-myc (de Benedetti *et al.*, 1994). Regulation of cap-binding activity is achieved in several ways including phosphorylation of eIF4E, phosphorylation of a protein which inhibits the interaction of eIF4E with eIF4G (see below) and by phosphorylation of eIF4G (Mader and Sonenberg, 1995). Whereas the mechanism by which phosphorylation of eIF4E and eIF4G affect cap-binding activity at the molecular level are poorly understood, we learned more recently how eIF4E activity is regulated by a group of proteins which interact with eIF4E, termed 4E-BPs (4E-binding proteins) (Lin *et al.*, 1994; Pause *et al.*, 1994). These proteins compete with eIF4G for interaction with eIF4E, interfere in this way with the formation of the eIF4F complex and thereby inhibit cap-dependent translation (Haghighat *et al.*, 1995). Their activity is regulated by phosphorylation. In adipose cells treated with insulin, 4E-BPs become phosphorylated and dissociate from eIF4E, thereby relieving translational inhibition (Pause *et al.*, 1994). The phosphorylation of 4E-BPs appears to be under the control of the FRAP/RAFT signal transduction pathway since treatment of NIH 3T3 cells with rapamycin results in inhibition of 4E-BP1 phosphorylation (Beretta *et al.*, 1996; von Manteuffel *et al.*, 1996).

In *S.cerevisiae* the eIF4F complex is composed of the cap-binding protein eIF4E and eIF4G/p150 (the homologue of mammalian p220). eIF4G/p150 is encoded by the genes *TIF4631* and *TIF4632*. They encode two proteins (eIF4G1 and eIF4G2) which are 53% identical at the amino acid sequence level. While deletion of one of the two genes does not severely affect growth, deletion of both is lethal for the cell (Goyer *et al.*, 1993).

The molecular interaction between eIF4E and eIF4G1 has been studied recently *in vitro* (Mader *et al.*, 1995). Studies with truncated proteins defined a region comprising amino acids 378–504 of yeast eIF4G1 as the minimal sequence necessary for binding to eIF4E. Very recently, a region located between amino acid residues 400 and 515 on yeast eIF4G2 has also been shown to bind eIF4E (A.Sachs, personal communication). A 12 amino acid motif conserved between mammalian and *S.cerevisiae* eIF4G was proposed to be critical for interaction with eIF4E. Interestingly, a related motif is also found in the amino acid sequence of mammalian 4E-BPs and point mutations in this motif abolish the interaction with eIF4E (Mader *et al.*, 1995).

In the yeast, in addition to eIF4G a small protein termed p20 (161 amino acids) is found associated with eIF4E (Altmann *et al.*, 1989a; Lanker *et al.*, 1992). The functional significance of this interaction remained unclear except that disruption of *CAF20*, the gene encoding p20, could be shown to stimulate cell growth on rich media (Lanker *et al.*, 1992). We show here, that p20 is an inhibitor of cap-dependent translation initiation which competes with eIF4G for binding to eIF4E.

Results

Sequence homology between p20 and eIF4G

The proteins eIF4G1 and p20 copurify with yeast eIF4E on m⁷GDP-Sepharose affinity columns (Altmann *et al.*, 1989a; Lanker *et al.*, 1992) and may therefore have related structural features which allow them to bind to eIF4E. We used the Bestfit program of the GCG package (University of Wisconsin) for amino acid sequence comparison of the two proteins. The overall identity between p20 (total of 161 amino acids) and yeast eIF4G1 (amino acids 448–647, Figure 1A and B) is 21% (34 identical amino acids). Interestingly, a 10 amino acid motif present in yeast eIF4G (sequence KYTYGPTFLL; amino acids 449–458 in eIF4G1 and amino acids 420–429 in eIF4G2), mammalian eIF4G (sequence KKRYDREFLL; amino acids 413–422; Mader *et al.*, 1995), wheat germ p82 subunit of iso4F (sequence RVRYSRDQLL; amino acids 63–72; Allen *et al.*, 1992) and mammalian eIF4E-BPs (sequence RII-YDRKFLL/M; amino acids 51–60; Pause *et al.*, 1994) is also present in p20 (sequence KYTIDELFQL; amino acids 3–12; Figure 1C). The consensus amino acid sequence motif forms part of the previously reported sequence necessary for binding of eIF4G to eIF4E (Mader *et al.*, 1995). These findings suggest that p20 may bind directly to eIF4E through a protein motif which is related to a motif in eIF4G.

elF4E binds to a 50 amino acid domain of elF4G and to p20 in vitro

To verify that the protein domain in eIF4G1 carrying the conserved sequence motif described above binds to eIF4E we produced truncation mutants of eIF4G1 as glutathione-S-transferase (GST) fusion proteins and tested their capability to bind eIF4E *in vitro*. The purified proteins are shown in Figure 2A. We found that the fusion protein GST-4G-9d (amino acids 159-490, Figure 1A; and Figure 2A, lane 4) carries the binding site for eIF4E because it is capable of specifically retaining eIF4E on a glutathione-Sepharose 4B resin (Figure 2B, lane 6). The source for eIF4E used in this assay was an extract from *Escherichia coli* cells that expresses eIF4E from the T7 promoter (Figure 2A, lanes 1 and 2). Further analysis of this region showed that a sequence comprising 50 amino acids of eIF4G1 is sufficient to bind eIF4E because the GST-4G-9d/441-490 fusion protein (amino acids 441-490, Figure 1A and Figure 2A, lane 3) was also capable of specifically retaining eIF4E on a glutathione-Sepharose 4B resin (Figure 2B, lane 5). In the absence of GST-4G fusion protein, very little eIF4E was retained by the resin (Figure 2B, lane 2). The GST-4G-11c fusion protein (amino acids 882-952) was not capable of binding eIF4E (data not shown). These results indicate that the yeast eIF4G1

Fig. 1. p20 and eIF4G share amino acid sequence homology. **(A)** Schematic representation of yeast eIF4G1 containing 952 amino acids (aa). The 331 amino acid fragment eIF4G-9d (aa 159–490), the eIF4E-binding domain on eIF4G-9d/441–490 (shadowed box) and the C-terminal fragment eIF4G-11c (aa 882–952) are indicated by arrows. **(B)** Alignment of amino acids 448–647 of eIF4G1 with the complete sequence of p20. The identity between both sequences is 21%. The amino acids were aligned using the program Bestfit from the GCG software package on a VAX computer. **(C)** Alignment of the conserved motif K/R/Y/T/R/YD/G/R/PXFL found in p20, eIF4G from different sources and mammalian 4E-BPs. The second most common amino acid at each position is indicated below the consensus motif. The alignment was done by eye.

region encoded by amino acids 441–490 is sufficient for specific interaction of eIF4G1 with the yeast cap-binding protein eIF4E *in vitro*.

To test whether p20 can bind eIF4E we fused the entire open reading frame of p20 in frame to GST and used the GST fusion protein (apparent molecular weight of 48kDa on SDS-polyacrylamide gel, Figure 2A, lane 5; and 2B, lane 1) to perform binding assays as described for the GST-eIF4G1 fusion proteins. The results demonstrate that the GST-p20 fusion protein bound to glutathione-Sepharose 4B is also capable of specifically retaining eIF4E (Figure 2B, lane 3).

We asked whether mutations in eIF4E such as the allele

C

Fig. 2. eIF4E binds to eIF4G and p20. (A) Proteins used in this study were fractionated by SDS-PAGE and stained with Coomassie Blue R-250. Lane 1, 30 µg extract of *E. coli* cells expressing eIF4E; lane 2, 1 µg affinity-purified eIF4E; lane 3, 100 ng GST-eIF4G-9d/441-490 fusion protein; lane 4, 1 µg GST-eIF4G-9d fusion protein; lane 5, 1 µg GST-p20 fusion protein (the faster migrating band is probably a degradation product); lane 6, 400 ng GST protein. (B) GST fusion protein purified from *E. coli* extracts (about 50 µg total protein) was bound to glutathione-Sepharose resin (see Materials and methods). After washing with buffer A, the resin was incubated with *E. coli* extract (50 µg total protein) containing 0.4 µg eIF4E, washed with buffer A and protein adsorbed to the matrix eluted by incubating with glutathione. Adsorbed protein was fractionated by SDS-PAGE and analyzed by Western blot immunodecoration with antiserum containing antibodies against eIF4E, eIF4G and p20. The Western blot is shown. Lane 1, GST-p20 fusion protein; lane 2, eIF4E extract; lane 3, eIF4E extract plus GST-p20 fusion protein; lane 4, GST-eIF4G-9d/441-490 fusion protein; lane 5, eIF4E extract plus GST-eIF4G-9d/441-490 fusion protein; lane 6, eIF4E extract plus GST-eIF4G-9d fusion protein. eIF4E migrates as a protein band of ~25 kDa. (C) *Escherichia coli* extract (50 µg total protein) expressing wild-type eIF4E (0.4 µg) or ts4-2 was incubated for 30 min at room temperature with 20 µl resin containing GST-p20 covalently attached to BrCN activated Sepharose. After washing with buffer A (+ 50 µg/ml BSA + 0.1% TX-100), protein adsorbed to the resin was eluted with 12 µl SDS sample buffer for 3 min at 95°C. Non-bound (NB; lanes 1 and 2) and bound eIF4E (B; lanes 3 and 4) were fractionated by SDS-PAGE and analysed by Western blotting with a polyclonal antibody against eIF4E (1:1000 dilution in 0.5% BSA).

ts4-2 which confers a temperature-sensitive phenotype to yeast (Altman *et al.*, 1989b) also affect the interaction of eIF4E with p20. eIF4E ts4-2 has reduced cap-binding activity and extracts from yeast cells carrying this mutant allele have been previously shown to be severely affected in their capacity to translate capped reporter mRNAs such as CAT (chloramphenicol acetyl transferase). As shown in Figure 2C, >50% of wt-eIF4E was adsorbed to GST-p20 covalently attached to Sepharose 4B (lane 3), while no ts4-2 protein was adsorbed (lane 4). This result clearly indicates that this allele of eIF4E has (in addition to reduced cap-binding activity) reduced p20-binding activity. This was exploited to test the effect of p20 on cap-dependent translation of different mRNAs *in vitro* (see below).

p20 and eIF4G compete for the same binding site on eIF4E

The presence of a conserved amino acid motif involved in eIF4E binding to p20 and eIF4G1 leads to the prediction

that both proteins may compete for the same binding site on eIF4E as shown recently for mammalian 4E-BP1 and eIF4G by *in vitro* experiments (Haghighat *et al.*, 1995). In order to study mutually exclusive binding of p20 or eIF4G1 to eIF4E, GST-p20 fusion protein (or GST by itself as a control; Figure 2A, lanes 5 and 6) was covalently attached to CNBr-activated Sepharose and purified eIF4E allowed to bind to it. Bound protein was eluted with SDS at 95°C and analysed by SDS-PAGE (polyacrylamide gel electrophoresis) and immunoblotting with an antibody against yeast eIF4E. Under the conditions chosen for this experiment, GST-p20 immobilized to CNBr-Sepharose was capable of specifically retaining ~50% of eIF4E (Figure 3A, lanes 3 and 4) while almost no eIF4E was retained by GST-Sepharose alone (lanes 1 and 2). Since eIF4E binds to immobilized GST-p20, we asked whether pre-incubation of eIF4E with other proteins could out-compete this interaction. Before adsorption to the immobilized GST-p20, eIF4E was pre-incubated with different purified GST fusion proteins. Interestingly, only equimolar

A

B

Fig. 3. p20 and eIF4G compete for eIF4E-binding *in vitro* and *in vivo*. (A) 20 pmol (0.5 µg) purified eIF4E was pre-incubated with buffer A or with equimolar amounts of GST or GST fusion proteins for 10 min at room temperature. The mixture was then added to 20 µl resin (GST covalently attached to Sepharose, lanes 1 and 2, or GST-p20 covalently attached to Sepharose, lanes 3–12) and further incubated for 30 min at room temperature. After washing with buffer A, protein adsorbed to the resin was eluted with 20 µl SDS sample buffer for 3 min at 95°C. Bound (B; lanes 1, 3, 5, 7, 9 and 11) and non-bound eIF4E (NB; lanes 2, 4, 6, 8, 10 and 12) were fractionated by SDS-PAGE and analysed by Western blotting with a polyclonal antibody against eIF4E (1:1000 dilution in 0.5% BSA). Lanes 1–4, eIF4E pre-incubated with buffer A; lanes 5 and 6, eIF4E pre-incubated with soluble GST-p20; lanes 7 and 8, eIF4E pre-incubated with soluble GST-eIF4G-9d; lanes 9 and 10, eIF4E pre-incubated with soluble GST; lanes 11 and 12, eIF4E pre-incubated with soluble GST-eIF4G-11c. (B) Immunoprecipitation of eIF4G and p20 from a total yeast cell extract with rat polyclonal antisera was performed as described in Materials and methods. The Western blots decorated with anti-eIF4G antibody (top), anti-eIF4E antibody (middle) and anti-p20 antibody (bottom) are shown. Lane 1, protein bound (B) to anti-eIF4G antibody; lane 2, non-bound (NB) fraction; lane 3, protein bound (B) to anti-p20 antibody; lane 4, non-bound (NB) fraction.

amounts of soluble GST-p20 (Figure 3A, lanes 5 and 6) or GST-4G-9d (Figure 3A, lanes 7 and 8) could efficiently outcompete the binding of eIF4E to immobilized GST-p20, while equivalent amounts of soluble GST (Figure 3A, lanes 9 and 10) or soluble GST-4G-11c (Figure 3A, lanes 11 and 12) could not exert a competitive effect. These data clearly indicate that p20 and eIF4G1 compete for binding to eIF4E.

In a different approach, eIF4G-9d or p20 was incubated with eIF4E and the resulting eIF4G-9d-eIF4E and p20-eIF4E complexes isolated on m⁷GDP-Sepharose. The complexes were then incubated with either GST-p20 or GST-eIF4G-9d bound to glutathione-Sepharose. Both, GST-p20 and GST-eIF4G-9d could retain eIF4E but not eIF4G-9d or p20 (results not shown). These results further support the conclusion that eIF4E exists as a complex with either p20 or eIF4G1 but not with both.

To determine whether mutually exclusive binding of eIF4G1 and p20 to eIF4E also occurs *in vivo* we performed co-immunoprecipitation experiments with yeast extracts derived from growing cells. As shown in Figure 3B, a polyclonal antiserum against yeast eIF4G specifically precipitates eIF4G and eIF4E from this yeast extract but not p20 (lane 1) while all three proteins are found in the supernatant (lane 2). However, when a polyclonal antiserum against p20 was used, eIF4E co-precipitated with p20 (lane 3) but not eIF4G which could be detected only in the supernatant (lane 4). These results demonstrate that two distinct complexes exist in the crude cell extract, namely, eIF4E in association with p20 or eIF4E bound to eIF4G.

p20 inhibits cap-dependent but not cap-independent translation

To investigate the effect of p20 on cap-dependent translation, we made use of a cell-free extract from the mutant yeast strain ts4-2 (Altmann *et al.*, 1989b) which has reduced eIF4E activity and supports efficient cap-independent but not cap-dependent translation. Upon addition of wild-type eIF4E, cap-dependent translation in the ts4-2 extract is fully reconstituted (Altmann *et al.*, 1989b). We compared the translational efficiency of two capped CAT mRNAs which differ only in their 5' untranslated region, one of them (Ω CAT mRNA) containing the 67 nucleotide Ω sequence derived from the 5' untranslated region of tobacco mosaic virus mRNA (Sleat *et al.*, 1987) which confers eIF4E-independent translation (Altmann *et al.*, 1990). Addition of CAT mRNA in the absence of exogenous eIF4E gave a 7-fold stimulation of translation (Figure 4A, assay 2) as compared with omission of CAT mRNA (assay 1). Addition of limiting amounts of purified eIF4E (1 pmol) gave a 10-fold stimulation of [³⁵S]methionine incorporation (assay 3) while addition of higher amounts of eIF4E (3 pmol) gave at least a 20-fold stimulation of incorporation (not shown). Note, that sub-optimal amounts of exogenous eIF4E were used in this experiment to avoid too high factor concentrations (mutant endogenous and exogenous factor). High concentrations of eIF4E would require even higher concentrations of p20 to sequester eIF4E. When GST-p20 (up to 0.4 pmol) was added in the absence of wild-type eIF4E a 2-fold inhibition of CAT-mRNA translation was observed (assay 5). This inhibitory effect became more drastic upon addition of wild-type eIF4E to the extract: protein synthesis almost dropped to basal levels (assay 7). Since the interaction between p20 and eIF4E ts4-2 protein *in vitro* is poor (Figure 2C), we assume that the stronger inhibitory effect of GST-p20 on CAT mRNA translation in the presence of wild-type eIF4E is due to the interaction of p20 with wild-type eIF4E and the binding of this complex to capped mRNA (Altmann *et al.*, 1989a).

Fig. 4. Translation in an eIF4E-dependent extract. *In vitro* translation assays in an extract derived from the temperature-sensitive strain eIF4E *ts4-2* (Altmann *et al.*, 1989b) were done at 20°C in 14 µl incubation mixtures programmed (A) with 150 ng ⁷mGpppG-CAT mRNA or (B) with 150 ng ⁷mGpppG-Ω-CAT mRNA. 5 µl aliquots were removed at the times indicated and [³⁵S]methionine incorporation measured. 1, □, no mRNA added; 2, ■, plus mRNA; 3, ○, plus mRNA, plus 1 pmol eIF-4E; 4, △, plus mRNA, plus 0.2 pmol GST-p20; 5, ▲, plus mRNA, plus 0.4 pmol GST-p20; 6, ●, plus mRNA, plus 1 pmol eIF-4E, plus 0.2 pmol GST-p20; 7, ⊕, plus mRNA, plus 1 pmol eIF-4E, plus 0.4 pmol GST-p20. Insets: 3.5 µl aliquots (after incubation for 30 min) were fractionated by SDS-PAGE and the gels prepared for autoradiography. The regions of autoradiograms with CAT are shown. The lane numbers in the insets (1–7) correspond to the numbers used in the [³⁵S]methionine incorporation assays.

A quite different result was obtained when Ω CAT mRNA was translated in the eIF4E-dependent extract (Figure 4B). As previously shown, Ω CAT mRNA translation is less efficient but is not dependent on addition of wild-type eIF4E (Altmann *et al.*, 1990). Increasing amounts of GST-p20 had only a mild inhibitory effect on

A

	Strain	Genotype	Transformed with	Generation time (min)
1.	YMA-2A	<i>caf20::URA3</i>	2µ-vector	112 ± 12
2.	"	"	2µ-CAF20	139 ± 8
3.	YMA-4B	wild type	2µ-vector	121 ± 8
4.	"	"	2µ-CAF20	153 ± 9

B

Fig. 5. Overexpression of p20 slows down cell growth. (A) The isogenic strains YMA-2A (*caf20::URA3* disruption) and YMA-4B (wild-type) were transformed with the 2µ vector Yep13 (2µ vector) or with the 2µ vector carrying the *CAF20* gene (2µ-CAF20). The generation time of cells growing in minimal medium (SD) supplemented with amino acids at 30°C was determined by cell counting with a Neubauer haemocytometer. The generation times and error margins presented were determined in three independent experiments. (B) p20 expression in cell lines described in (A). Approximately 20 µg (lanes 1–3) or 15 µg (lane 4) of total protein from exponentially growing cells was fractionated by SDS-PAGE and analyzed by Western blot immunodecoration with a polyclonal antibody against p20.

Ω CAT mRNA translation in the presence or absence of added eIF4E (assays 6 and 7), indicating that cap-independent translation is much less inhibited by the p20-eIF4E complex.

All incorporation data were confirmed by SDS-PAGE analysis of the CAT synthesized from both mRNAs (insets in Figure 4A and B). These data demonstrate that p20 is capable of specifically inhibiting cap-dependent translation in yeast.

The expression level of p20 affects cell growth

We reported previously that deletion of the gene encoding p20 (*CAF20*) reduces the generation time of exponentially growing cells as compared with the isogenic wild-type cells (Lanker *et al.*, 1992). Since p20 inhibits cap-dependent mRNA translation *in vitro*, we tested whether overexpression of p20 has an inhibitory effect on cell growth. We transformed both isogenic strains YMA-4B (wild-type) and YMA-2A (*caf20::URA3*-deletion) with a yeast multicopy plasmid carrying the entire *CAF20* gene (Altmann *et al.*, 1989a) and measured growth of transformed cells on minimal growth medium supplied with 2% glucose and amino acids. As summarized in Figure 5A, yeast strains transformed with the *CAF20* gene show a reduced rate of cell growth. Exponentially growing wild-

type cells (strain YMA-4B) and *CAF20* gene-deleted cells (strain YMA-2A) show an increase in generation time of 27–32 min when the level of p20 is raised by expressing it from a 2 μ vector. We have checked the copy-number of the 2 μ vector and the *CAF20* construct and found it to be similar in the different cell lines (results not shown), therefore the inhibitory effect on growth cannot be ascribed to different plasmid copy-numbers in the different cell lines.

The moderate inhibitory effect of p20 under these conditions correlates with the moderate level of overexpression of p20 (Figure 5B). Western blot analysis of total protein from the strains used in the experiment showed that overexpression of p20 was only ~2-fold. Two protein bands of p20 are regularly detected. We speculate that the upper band might correspond to a phosphorylated form of the protein.

Discussion

Yeast translation initiation factor eIF4E interacts simultaneously with the cap structure of mRNA and with eIF4G. We believe that this interaction is a prerequisite for the binding of the 43S-preinitiation complex close to the 5' end of the mRNA: the eIF4E–eIF4G complex stimulates RNA secondary structure unwinding of the ribosome-binding site by the initiation factors eIF4A and eIF4B, and eIF4G binds the 43S-preinitiation complex by interacting with eIF3 on the surface of the small ribosomal subunit (Lamphear *et al.*, 1995). A fragment of eIF4G containing the amino acids 378–504 was reported earlier (Mader *et al.*, 1995) to bind eIF4E. We show here that a shorter region of 50 amino acids on eIF4G (amino acids 441–490) is sufficient to bind eIF4E in our *in vitro* experiments. This region contains an amino acid motif conserved in eIF4G from several species and in mammalian eIF4E-BPs (Mader *et al.*, 1995). This motif was recently shown to mediate the competition between eIF4E-BP1 and mammalian eIF4G (Haghighat *et al.*, 1995). Interestingly, the yeast protein p20 carries a similar motif at its amino terminus (Figure 1) making this sequence a strong candidate for an essential part of the eIF4E interaction domain. The binding of p20 and eIF4G to eIF4E is mutually exclusive *in vitro* (Figure 2B and 3A) as well as *in vivo* (Figure 3B) indicating that p20 probably fulfills a similar function in translation initiation as the mammalian eIF4E-BPs. Indeed, p20 is capable of specifically inhibiting cap-dependent translation initiation *in vitro* (Figure 4) while cap-independent translation is hardly affected at all. While this means that the interaction of eIF4E with eIF4G is not required for translation of certain mRNAs, it does not mean that eIF4G is dispensable. Experiments with rabbit reticulocyte lysate have shown that eIF4G is also required for cap-independent translation but that a C-terminal fragment of the factor lacking the eIF4E-binding domain can fulfill this function (Ohlmann *et al.*, 1995, 1996). For the majority of cellular mRNAs, however, the eIF4E–eIF4G interaction is probably very important and its regulation by small proteins of central physiological significance, e.g. in mammalian cells stimulated for growth (Lin *et al.*, 1994; Pause *et al.*, 1994). Insulin and growth factors stimulate cap-dependent initiation of translation in mammalian cells through the FRAP/RAFT signal trans-

duction pathway (von Manteuffel *et al.*, 1996). This leads to phosphorylation of eIF4E-BPs and enhanced eIF4E–eIF4F interaction. The drug rapamycin interferes with FRAP/RAFT function and, in this way, inhibits phosphorylation of eIF4E-BPs (Beretta *et al.*, 1996).

In the yeast *S.cerevisiae*, the eIF4E–eIF4G interaction is probably of similar importance and perhaps similarly regulated. Cells with a deletion of *CAF20* gene have a shorter generation time during exponential growth than isogenic wild-type cells (Lanker *et al.*, 1992; Figure 5 in this paper) suggesting that even in fast growing cells protein synthesis is partly inhibited by p20. On the other hand, overexpression of p20 in wild-type cells causes slower growth. Immunoblot experiments revealed that wild-type yeast cells contain ~1 mol eIF4E and 1 mol p20 per mol ribosomes but only ~0.1 mol eIF4G per mol ribosomes (our unpublished results). This indicates that eIF4G has a higher affinity for eIF4E than p20 and may explain the moderate effect of a 2-fold overexpression of p20 on cell growth. It also suggests that modification of eIF4G or p20 may be required to change the eIF4E–eIF4G interaction in wild-type cells. Indeed, p20 was shown to be a phosphoprotein (Zanchin and McCarthy, 1995) and therefore its activity could be modulated through phosphorylation as demonstrated for mammalian eIF4E-BPs (Lin *et al.*, 1994; Pause *et al.*, 1994).

At present, we do not know the physiological significance of p20 as a negative regulator of cap-dependent translation initiation. Since cells lacking p20 have a higher budding index than wild-type cells (our unpublished results) we suspect that p20 slows progression through the G₁ phase of the cell cycle. During this phase it may help to modulate the rate of protein synthesis in response to extracellular signals such as the availability of nutrients. This model is supported by the observation that reduction of eIF4E activity in yeast cells leads to arrest of growth in the G₁ phase and preferential translation of mRNAs which do not require eIF4E such as *UBI-4* mRNA (Brenner *et al.*, 1988). The signal transduction pathway leading to changes in the eIF4E–eIF4G interaction in yeast may be similar to the one in higher eukaryotic cells since treatment of yeast cells with the drug rapamycin leads to arrest of growth in the G₁ phase and phenotypic resemblance to starved cells (Barbet *et al.*, 1996). Further experiments will elucidate the physiological role played by p20 in the yeast *S.cerevisiae*.

Materials and methods

Manipulation of yeast cells

Yeast cells were transformed using the lithium acetate method (Ito *et al.*, 1983).

DNA manipulations

Unless indicated otherwise, DNA manipulations were carried out according to Sambrook *et al.* (1989) using *E.coli* strain XL2B (Stratagene) for subclonings and maintenance of plasmid DNA.

Plasmids and vector construction

For expression of GST fusion proteins in *E.coli* the vector pGEX-11ambdaT (Pharmacia) was chosen. eIF4G constructs were derived from the vector pCG8.4 which carries the complete *TIF4631* open reading frame on a 3266 bp cDNA cloned into *EcoRI* site of Bluescript (Goyer *et al.*, 1993). For expression of eIF4G-9d a 996 bp *Bgl/III* DNA fragment (comprising codons 159–490) was subcloned in the proper orientation

into *Bam*HI-restricted pGEX-11ambdaT vector. For expression of eIF4G-9d/441–490 a 150 bp *Eco*RV–*Eco*RI DNA fragment (comprising codons 441–490) from eIF4G-9d was subcloned into *Sma*I–*Eco*RI double-restricted pGEX-11ambdaT vector. For expression of eIF4G-11c a 400 bp *Bgl*III–*Eco*RI DNA fragment (comprising codons 882–952) was subcloned into *Bam*HI–*Eco*RI restricted pGEX-11ambdaT vector.

The clone Yep13-1.2 (Altmann *et al.*, 1989a) carrying the entire *CAF20* gene was used as a template for PCR amplification with the 5' primer NS3 (CCGGATCCATCAAGTATACTATCGATGAG) and the 3' primer EK4 (CCAAGCTTGAATTCCTTAATGGTGATGGTGATGGTG-GCTTCGTCGTCCTTCG). The resulting DNA fragment contained at its 5' end a *Bam*HI restriction site (underlined in NS3) followed by the second codon and the entire open reading frame of *CAF20* and an *Eco*RI restriction site (underlined in EK4). The amplified DNA fragment was cut with *Bam*HI and *Eco*RI and ligated into *Bam*HI–*Eco*RI double-restricted pGEX-11ambdaT vector.

Purification of GST fusion proteins

Escherichia coli extract from cells expressing either GST–p20 or GSTeIF-4G-9d fusion protein was prepared by inducing 1 l cultures (in LB medium) of exponentially growing cells at an A_{600} of 0.6–0.8 for 2 h with 0.5 mM isopropyl-thio-galactoside (IPTG). Cells were harvested and resuspended in 10 ml PBS (Phosphate Buffered Saline: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.3) and lysed with the help of a French Press (at 10 000 p.s.i.). The suspension was centrifuged at 12 000 g for 10 min and the cleared lysate incubated with 1 ml glutathione–Sephadex 4B resin (Pharmacia) overnight at 8°C. The resin was washed with 100 ml PBS at 8°C and bound protein eluted by incubating the resin with 1 ml of 10 mM reduced glutathione in buffer A (20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM MgCl_2) for 30–60 min at room temperature.

Binding assays on glutathione resin

GST fusion proteins purified from *E. coli* extracts (~50 µg total protein) were bound to 20 µl of glutathione–Sephadex 4B resin (Pharmacia) for 30 min at room temperature. The resin was then washed with buffer A and incubated for 30 min at room temperature with 0.4 µg eIF4E (from an *E. coli* extract; 50 µg total protein). *Escherichia coli* extract was centrifuged at 20 000 g for 15 min prior to incubation with resin in order to avoid non-specific adsorption of denaturated and/or aggregated protein. The resin was washed with buffer A and protein adsorbed to the matrix eluted by incubating with 10 mM reduced glutathione in buffer A for 15 min at room temperature.

Covalent coupling of GST fusion proteins to Sepharose and competition assays

Coupling of GST fusion proteins to CNBr-activated Sepharose was done according to the recommendations of the manufacturer (Pharmacia). 100 µg protein was bound to 1 ml CNBr–Sepharose (50% slurry, resuspended in 100 mM NaHCO_3 , pH 8.3, 500 mM NaCl) by incubating for 2 h at room temperature. Coupling efficiency was >90%. Protein covalently bound to Sepharose was kept in 1 ml buffer A with 0.005% NaN_3 at 4°C for ~2 weeks.

Competition assays were done in buffer A with 50 µg/ml bovine serum albumin (BSA) and 0.1% Triton X-100. 20 pmol eIF4E was pre-incubated with equimolar amounts of competitor protein (GST, GST–p20, GST–eIF4G-9d or GST–eIF4G-11c) for 10 min at room temperature. 15 µl of Sepharose–GST or Sepharose–GST–p20 was added to the incubation mixture and incubation continued for 30 min at room temperature. The resin was recovered by centrifugation at 2000 g for 5 min, washed twice with 100 µl buffer A and proteins bound to the resin eluted with 10 µl SDS sample buffer at 95°C for 3 min. Resin-bound and non-bound proteins were fractionated on a 15% SDS–polyacrylamide gel and identified by Western blot immunodecoration with a polyclonal antibody against eIF4E (1:1000 dilution in 0.5% BSA).

Immunoprecipitations

75 µl total yeast extract from strain CWO4 was incubated with 6 µl anti-eIF4G polyclonal antibody (rat serum) or anti-p20 antibody (rat serum) for 3.5 h at 0°C. 60 µl protein G–Sepharose [Pharmacia; pre-equilibrated in TBS (20 mM Tris–HCl, pH 7.5, 100 mM NaCl)] was added to the antibody–yeast extract mixtures and incubation continued at 0°C for 30 min with occasional mixing. Tubes were centrifuged at 2000 g for 5 min and the resin was washed twice with 500 µl TBS. Proteins were eluted with SDS-sample buffer for 3 min at 95°C and analysed by 15% SDS–PAGE and Western blotting. The blots were

decorated with rat polyclonal antibodies against eIF4E, eIF4G and p20 (1:1000 dilutions in 0.5% BSA).

Preparation of polyclonal rat antibodies

Rats were injected intraperitoneally with 60 µg fusion protein GST–eIF4G-9d in 50% complete Freund's adjuvant. The injection was repeated twice with incomplete Freund's adjuvant at intervals of 10 days. Rats were killed by cervical dislocation and blood was collected from the heart and centrifuged; serum was stored in aliquots at –70°C.

In vitro translation

Extracts for *in vitro* translation were prepared and cell-free translation performed as described earlier (Altmann *et al.*, 1985, 1989b).

SDS–PAGE and Western blot analysis

SDS–PAGE with 15% gels (Anderson *et al.*, 1973) and Western blotting were performed as described (Towbin *et al.*, 1979). Transfer of proteins to nitrocellulose was for 30 min at 60 V in a Mini Trans Blot Cell (Bio-Rad). Blots were saturated with TBS containing 2.5% BSA for 5 min at room temperature and incubated overnight at room temperature with rat polyclonal antibodies (1:1000 dilutions in TBS containing 0.5% BSA). After washing with TBS the blots were decorated for 1 h with rabbit anti-rat antibody conjugated to peroxidase (DAKO, Denmark) and stained with 0.018% chloronaphthol, 0.006% H_2O_2 in TBS.

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