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IRP-1 Binding to Ferritin mRNA Prevents the Recruitment of the Small Ribosomal Subunit by the Cap-Binding Complex eIF4F

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Summary

Binding of iron regulatory proteins (IRPs) to IREs located in proximity to the cap structure of ferritin H- and L-chain mRNAs blocks ferritin synthesis by preventing the recruitment of the small ribosomal subunit to the mRNA. We have devised a novel procedure to examine the assembly of translation initiation factors (eIFs) on regulated mRNAs. Unexpectedly, we find that the cap binding complex eIF4F (comprising eIF4E, eIF4G, and eIF4A) assembles even when IRP-1 is bound to the cap-proximal IRE. This assembly is futile, because bridging interactions between eIF4F and the small ribosomal subunit cannot be established in the presence of IRP-1. Our findings provide insight into translational control by an mRNA binding protein at the level of translation initiation factors and uncover a key regulatory step in iron homeostasis.

Introduction

The iron storage protein ferritin protects cells from the damaging effects of excess free iron (Britton et al., 1994; Halliday et al., 1994). High iron levels stimulate ferritin synthesis, while ferritin expression is translationally repressed in iron-deficient cells and in cells exposed to oxidative stress or nitric oxide. Control is exerted by the binding of iron regulatory protein-1 (IRP-1) or IRP-2 to an iron-responsive element (IRE) near the cap structure of ferritin mRNAs (reviewed in Hentze and Kühn, 1996; Rouault et al., 1996). This regulatory interaction is perturbed in the dominantly inherited hyperferritinemia/cataract syndrome, where point mutations in the IRE of ferritin L-chain mRNA prevent IRP binding and uncouple ferritin L-chain synthesis from translational control (Beaumont et al., 1995; Girelli et al., 1995).

While translational control is common in early development, cell differentiation, and metabolic regulation, much remains to be learned about the underlying mechanisms. For translational control by mRNA-specific regulatory proteins, the IRE/IRP system is currently one of the best understood: the translational regulation of ferritin mRNAs by IRPs was recapitulated in cell-free translation systems from rabbit reticulocytes with in vitro transcribed, capped indicator mRNA and recombinant human IRP-1 (Gray et al., 1993). Using sucrose gradient analyses, IRP-1 binding to the ferritin IRE was shown to prevent the recruitment of the small ribosomal subunit to the message (Gray and Hentze, 1994). A cap-proximal position of the IRE is important to enact this mechanism, as cap distantly (>60 nucleotides) located binding sites fail to prevent 43S complex recruitment (Paraskeva et al., unpublished data) leading to an impairment of translational control (Goossen and Hentze, 1992).

To understand the molecular steps underlying IRP-regulated translational control, we investigated how the IRE/IRP complex affects the sequential binding of translation initiation factors to the mRNA. To this end, we devised the translation intermediate purification assay (TIP assay), a novel approach to study the assembly of translation initiation factors on mRNAs. We report that the full cap binding complexes eIF4F and eIF4B assemble even when IRP-1 is bound to the cap-proximal IRE. The TIP assay reveals that subsequent bridging interactions to the small ribosomal subunit cannot be established, providing an explanation for the inhibition of 40S subunit recruitment.

Results and Discussion

Establishment and Validation of the Translation Intermediate Purification (TIP) Assay

An alternate approach needed to be devised to study the assembly of translation initiation factors on the IRE-regulated mRNA in the presence and absence of IRP-1. Based on procedures initially employed to examine the splicing process (Lamond and Sproat, 1994), we developed the translation intermediate purification (TIP) assay (Figure 1). In principle, a short biotinylated antisense 2′O-allyl oligoribonucleotide was annealed to the open reading frame of an mRNA, which is translationally regulated by a cap-proximal IRE (WT-IRE). The 2′O-allyl modification of the antisense oligonucleotide was chosen to increase the stability of the resulting mRNA/oligonucleotide hybrid, to avoid RNA degradation by RNase H activities, and to prevent RNA helicases from unwinding the mRNA/oligonucleotide hybrid (Lamond and Sproat, 1994). Subsequently, translation initiation factors and ribosomes were allowed to assemble on the mRNA in a cell-free translation reaction, prior to binding of the mRNA/oligonucleotide hybrid and its assembled translation initiation intermediates to a macroporous metacrylate polymer matrix coupled to streptavidin. Following several washes, bound proteins and RNAs were eluted, and ribosomal subunit association was assessed by probing for the 18S and 28S rRNAs. The binding of translation initiation factors was determined by immunoblotting with specific antibodies. ^{32}P-labeling of the CAT indicator mRNA templates served as a recovery control.

The validity of the TIP assay was ascertained by analysis of translation initiation reactions in which ribosome assembly was systematically perturbed by the use of pharmacological inhibitors (Figure 2A, left panel). For

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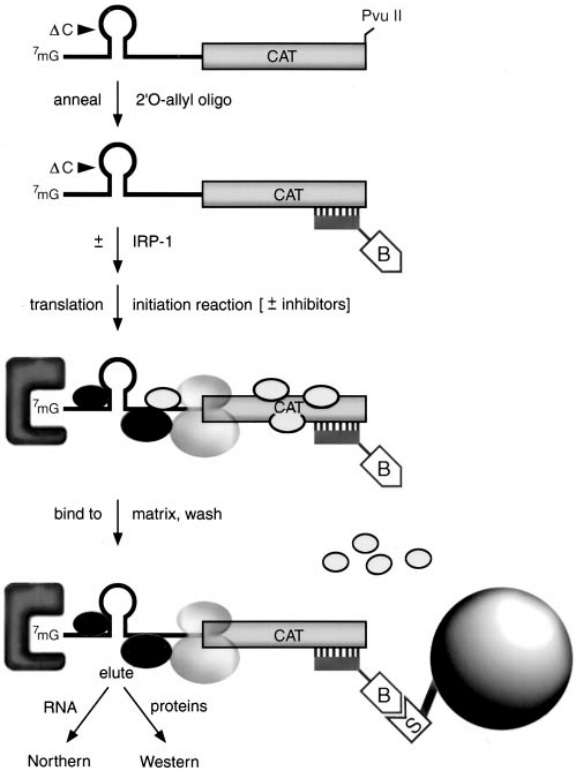

Figure 1. Schematic Outline of the Translation Intermediate Purification (TIP) Assay

The human ferritin H-chain IRE-regulated CAT indicator construct is identical to that used in earlier studies (Gray and Hentze, 1994). The ΔC mutation results from the deletion of the first nucleotide of the 5′-CAGUGC-3′ IRE loop and drastically reduces IRP-1 binding. B, biotin (the oligo carries three biotinylated nucleotides at its 5′ end); S, streptavidin.

comparison, initiation intermediates were analyzed on sucrose gradients (Figure 2A, right panel). Cycloheximide interferes with the peptidyl transfer reaction and thus arrests 80S ribosomes at the AUG initiator codon (open squares in the sucrose gradient analysis [Obrig et al., 1971]). Since GTP hydrolysis is required prior to the joining of the 60S ribosomal subunit with the 40S subunit, the nonhydrolyzable GTP analog GMP-PNP induces the accumulation of mRNAs where small ribosomal subunits are stalled at the initiation codon (closed diamonds [Hershey and Monro, 1966; Anthony and Merrick, 1992]). In the presence of cap analog, the binding of the small ribosomal subunit to the mRNA is prevented, and ribosome-free messenger ribonucleoprotein particles (mRNPs) accumulate (closed squares). In the TIP assay, the binding of ribosomal subunits to the mRNA is analyzed by Northern blotting for 18S rRNA (representing the binding of the 40S ribosomal subunit) and 28S rRNA (representing the binding of the 60S ribosomal subunit). As shown in Figure 2A (left panel), 18S and 28S rRNA hybridization signals were obtained exactly as expected from sucrose gradient analysis. Background binding (lane 4) was very low for the 28S rRNA and low for the 18S rRNA. Consistent and equal recovery of CAT mRNA was obtained under all conditions (Figure 2A, left panel, lanes 1–3).

We next evaluated IRP-1-mediated translational control by the TIP assay, using either a WT-IRE mRNA or an mRNA with a mutated IRE (ΔC-IRE, see Figure 1) that displays drastically reduced IRP-1 binding (Rouault et al., 1988; also compare lanes 2 and 7 in Figure 4). Sucrose gradient analyses reproduced our earlier findings (Gray and Hentze, 1994) that IRP-1 acts by preventing the recruitment of the small ribosomal subunit to the WT-IRE mRNA but not to the ΔC-IRE mRNA (Figure 2B, right panel). The TIP assay reflected and thus confirmed this result, in that only a weak signal was obtained for the 18S rRNAs and 28S rRNA with WT-IRE mRNA in the presence of IRP-1 (Figure 2B, lane 4); by contrast, a strong signal was seen when IRP-1 was omitted (Figure 2B, lanes 1 and 3), or when ΔC-IRE mRNA was translated in the presence of IRP-1 (Figure 2B, lane 2). CAT mRNA recovery was equal (Figure 2B, lanes 1–4), and background binding of ribosomes in the absence of mRNA (lane 5) was again low. The TIP assay thus permits a gentle analysis of bona fide translation initiation intermediates in general, and of IRE/IRP-mediated translational regulation in particular.

Assembly of the Cap-Binding Complex eIF4F and eIF4B in the Presence of IRP-1

During the translation initiation process, cap-mediated recruitment of the small ribosomal subunit requires binding of eIF4E to the ^{7}mGpppN cap structure and bridging protein–protein interactions (Hentze, 1997; Morley et al., 1997; Sachs et al., 1997; see also Figure 5, upper panel): the N-terminal domain of eIF4G binds to eIF4E, while the central part of eIF4G interacts through eIF3 with the 40S subunit (Lamphear et al., 1995; Mader et al., 1995; Ohlmann et al., 1995). In addition, eIF4A and eIF4B must be recruited to the mRNA. eIF4A has been reported to bind to the central and the C-terminal region of eIF4G (Lamphear et al., 1995; Imataka and Sonenberg, 1997), and in conjunction with eIF4B to serve as an ATP-dependent RNA helicase important for ribosome binding (Rozen et al., 1990; Pause and Sonenberg, 1992; Altmann et al., 1993). Since the IRE must be located in proximity to the cap structure for IRP-1 to repress translation efficiently (Goossen and Hentze, 1992) and prevent 40S recruitment (Paraskeva et al., unpublished data), we speculated that the cap-proximal IRE/IRP-1 complex might inhibit the binding of the eIF4F complex (eIF4E, eIF4G, and eIF4A) to the mRNA.

WT-IRE and ΔC-IRE mRNAs were examined by the TIP assay in the presence or absence of IRP-1. Bound translation initiation factors were eluted from the mRNAs, and the Western blots were probed with antibodies directed against eIF4A, 4B, 4E, and 4G. As a control for ribosome binding, human autoantibodies against the 60S ribosomal subunit–associated P0 protein were used. As expected, the four initiation factors (eIF4A, 4B, 4E, and 4G) assembled on the ΔC-IRE mRNA in the presence of cycloheximide or GMP-PNP (Figure 3, lanes 5 and 6). Importantly, none of the translation initiation factors displayed significant mRNA binding in the presence of cap analog (Figure 3, lanes 4–6), showing that binding is specific and cap-mediated. In contrast, binding of the P0 protein was only seen under conditions that allow

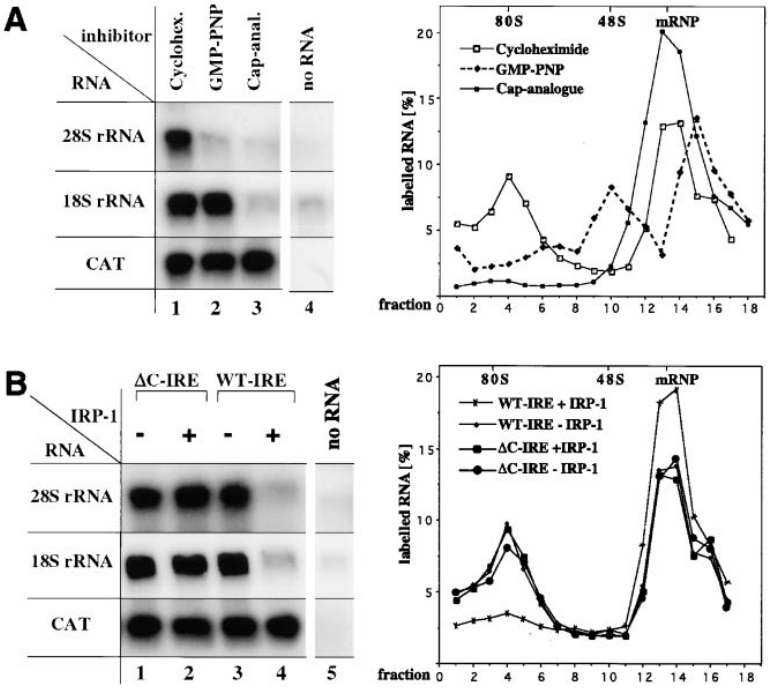

Figure 2. Validation of the TIP Assay

(A) Translation initiation assays were performed with ^{32}P-labeled ΔC-IRE mRNA in the presence of cycloheximide (lane 1), GMP-PNP (lane 2), or ^{7}mGpppG (lane 3), or no RNA was added (lane 4). The resulting translation initiation intermediates were analyzed by the TIP assay (left panel) and by linear sucrose gradient centrifugation (right panel).

(B) Initiation assays were carried out with ^{32}P-labeled ΔC-IRE (lanes 1 and 2) or WT-IRE mRNA (lanes 3 and 4) with (lanes 2 and 4) or without (lanes 1 and 3) recombinant IRP-1, or without RNA (lane 5). The resulting translation initiation intermediates were analyzed by the TIP assay (left panel) and by sucrose gradient centrifugation (right panel). ^{32}P-labeled CAT mRNA was detected directly. For the sucrose gradients, the labeled mRNA in each fraction is expressed as a percentage of total counts recovered. The sedimentation of 80S and 48S complexes and mRNPs is indicated.

the binding of the 60S ribosomal subunit (Figure 3, lane 5). The expected array of initiation factors and the P0 protein was also recovered bound to the ΔC-IRE mRNA in the presence of IRP-1, demonstrating that IRP-1 does not inhibit ribosome binding nonspecifically on a mutant IRE mRNA (Figure 3, lane 7). Surprisingly, IRP-1 permitted the unaffected assembly of eIFs 4A, 4B, 4E, and 4G even on WT-IRE mRNA (Table 1 and Figure 3, lane 2; note that the signal for the P0 protein as a positive control for the inhibitory effect of IRP-1 on ribosome recruitment was reduced to near background levels). This unexpected result is consistent with two additional observations. First, a capped WT-IRE mRNA is coprecipitated by anti-eIF4E antibodies both in the presence and the absence of IRP-1 (data not shown). Second, the IRP-1/IRE-WT mRNA complex is "supershifted" in an electrophoretic mobility shift assay by addition of recombinant eIF4E. This supershift requires capping of the RNA probe (data not shown). We therefore conclude that the WT-IRE mRNA assembles the complete cap binding complex eIF4F and eIF4B under conditions where its translation is repressed by a cap-proximal IRE/IRP-1 complex. Nonetheless, translation initiation fails to proceed toward recruitment of the small ribosomal subunit.

Table 1. Quantitative Analysis of eIF4F and eIF4B Assembly on IRP-1-Repressed mRNA

eIF	WT-IRE +IRP-1 (%)	ΔC-IRE +IRP-1 (%)	no RNA (%)
eIF4A	107 ± 7	103 ± 8	7 ± 5
eIF4B	103 ± 12	103 ± 10	6 ± 5
eIF4E	104 ± 17	106 ± 23	9 ± 6
eIF4G	101 ± 5	97 ± 6	10 ± 4
P0 (60S)	10 ± 6	103 ± 5	7 ± 2

TIP assays were performed as described in the legend to Figure 3. Binding of eIFs to WT-IRE and ΔC-IRE mRNAs was quantitated using the NIH image software (National Institutes of Health, USA). The signals obtained with the WT-IRE and ΔC-IRE mutant mRNAs in the absence of IRP-1 (Figure 3, lanes 1 and 5) were taken as 100%, and the effect of IRP-1 (lanes 2 and 7, respectively) and background binding (lane 3) expressed as percentages thereof. The experimental values and the standard deviation from three independent experiments are given.

IRP-1 Binding to the IRE Prevents Interactions between eIF4F/eIF4B and the Small Ribosomal Subunit–Associated eIF3

To understand further the IRP-induced blockage in 40S subunit recruitment, we tested whether eIF3, which participates in the recruitment of the small ribosomal subunit by means of interaction with both the central part of eIF4G and the 40S ribosomal subunit, was assembled into the IRP-1 containing mRNP. Western analysis showed that the binding of the 110/115 kDa and the 47 kDa subunits of eIF3 was reduced close to background levels when IRE-WT translation was repressed by IRP-1 (Table 2 and Figure 4, lanes 1–3). Similar results were obtained for the 170 kDa subunit of eIF3 (data not shown). Importantly, this mRNP retained the initiation factors of the eIF4 group (Figure 3) and IRP-1 (Figure 4, lane 2), indicating that mRNP proteins did not dissociate nonspecifically during purification. The positive controls in lanes 5 and 6 demonstrate that eIF3 was recovered by the TIP assay under conditions where translation was not blocked by IRP-1. The mere presence of IRP-1 (without high-affinity binding to the mRNA) did not inhibit eIF3 binding (lane 7; Table 2). Thus, IRP-1 prevents interactions between eIF4F/eIF4B and the small ribosomal subunit (Figure 5), such as the eIF4G–eIF3 interaction (Lamphear et al., 1995; Mader et al., 1995; Ohlmann

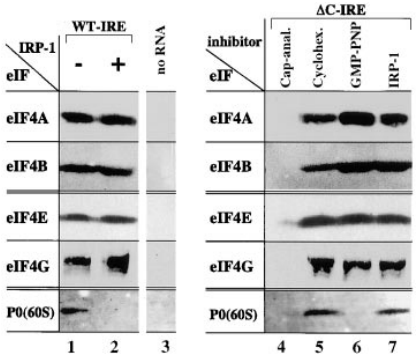

Figure 3. Assembly of the Cap-Binding Complex eIF4F and eIF4B on IRP-1-Repressed mRNA

TIP assays were performed with WT-IRE mRNA in the absence (lane 1) or the presence (lane 2) of IRP-1; without mRNA (lane 3); or with the ΔC-IRE mutant mRNA in the presence of ^{7}mGpppG (lane 4), cycloheximide (lane 5), GMP-PNP (lane 6), or IRP-1 (lane 7). Proteins were eluted from the streptavidin beads after washing and analyzed by Western blotting using antibodies directed against eIF4A (46 kDa), eIF4B (80 kDa), eIF4E (25 kDa), eIF4G (220 kDa), and the 60S subunit–associated phosphoprotein P0 (37 kDa).

et al., 1995) and the interaction between mRNA-bound eIF4B and eIF3 (Methot et al., 1996). This effect is specific and requires the binding of IRP-1 to the IRE.

The binding of the cap-binding complex eIF4F and of eIF4B to the mRNA in the presence of IRP-1 was unexpected, because an IRE/IRP-1 complex must be located in proximity (within 40 nucleotides) to the cap structure to repress translation efficiently (Goossen and Hentze, 1992) and to inhibit 40S subunit recruitment (Paraskeva et al., unpublished data). The binding of this complex may, however, have physiologically important implications. First, eIF4F may poise the mRNA for translational activation when IRP activity is diminished following an increase in cellular iron levels. Second, the association of eIF4F with translationally repressed WT-IRE mRNA may help to maintain the stability of the mRNA, because the bound eIF4F complex may protect it from decapping and subsequent exonucleolytic decay (Beelman and

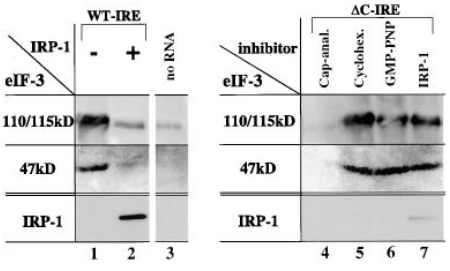

Figure 4. IRP-1 Prevents eIF3 Binding

The TIP assay was performed as described in the legend of Figure 3. The Western blot was probed with an antiserum against eIF3, and subsequently with an IRP-1 antibody. Signals corresponding to the 110/115 kDa eIF3 subunits and the 47 kDa subunit are shown. As the 110/115 kDa doublet is difficult to resolve by SDS PAGE and immunoblotting (Asano et al., 1997), we cannot determine if the 110/115 kDa signal represents either one or both of these eIF3 subunits.

Table 2. Quantitative Analysis of the Inhibitory Effect of IRP-1 on eIF3 Binding

eIF3	WT-IRE +IRP-1 (%)	ΔC-IRE +IRP-1 (%)	no RNA (%)
110/115 kDa	24 ± 12	111 ± 22	19 ± 7
47 kDa	11 ± 8	105 ± 6	8 ± 5

TIP assays were performed as described in the legend to Figure 4 and quantitatively evaluated as described in the legend to Table 1.

Parker, 1995). Our results also indicate that the recruitment of eIF4G, eIF4A, and eIF4B in the presence of IRP-1 requires eIF4E binding to the cap structure, because the inhibitory effect of cap analog on eIF4G, eIF4A, and eIF4B binding is comparable to its direct inhibitory effect on eIF4E binding to the mRNA (Figure 3, compare lanes 2, 4, and 5; data not shown). In addition to providing insight into the mechanism of translational control by IRP-1, our findings also add information on an unresolved issue in translation: the kinetic order in which eIF4G interacts with eIF4E and eIF3, respectively (Pain, 1996). While some investigators have proposed that eIF4G first associates with eIF4E before contacting eIF3 bound to the 40S subunit (Hershey, 1991; Merrick, 1992; Haghighat and Sonenberg, 1997), others have argued that eIF4G must first bind to the eIF3/40S complex and then direct this ribosomal complex to the mRNA by binding to eIF4E at the cap structure (Joshi et al., 1994; Rhoads et al., 1994; Rau et al., 1996). Our results support the former kinetic model where eIF4G binds to eIF4E independently of an interaction with eIF3 (Figure 3).

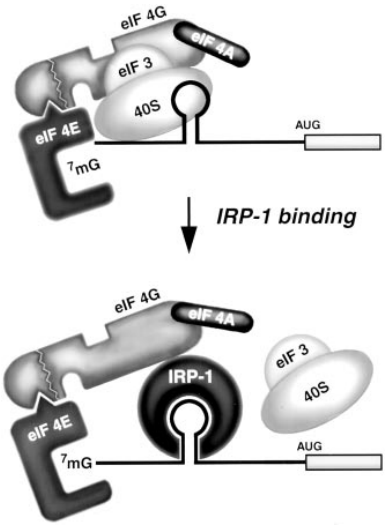

Figure 5. Translational Regulation by IRP-1

(Upper panel) Ribosome recruitment via the cap structure involves the depicted interactions from the cap binding protein eIF4E via eIF4G and eIF3 to the small ribosomal subunit.

(Lower panel) The data presented in this manuscript reveal that translational inhibition by IRP-1 binding to the IRE permits eIF4F (eIF4E, eIF4G, and eIF4A) assembly but prevents the recruitment of eIF3/40S subunit complexes. The bound eIF4B is not depicted to avoid misleading implications regarding its interaction partners. We also do not intend to imply a 1:1 stochiometry between eIF4G and eIF4A binding.

Why does the small ribosomal subunit fail to be recruited in spite of the successful preassembly of the eIF4F complex as well as the eIF4A/eIF4B helicase on the mRNA? Our results show that interactions between eIF4G and eIF3 cannot be established in the presence of IRP-1 (Figure 4). This interaction has been implicated by several laboratories to make an important contribution to 40S recruitment (Lamphear et al., 1995; Mader et al., 1995; Ohlmann et al., 1995), although its critical nature for this process has not yet been proven. Figure 4 demonstrates that IRP-1 can only do so when bound to the IRE. Moreover, even a high excess of free IRP-1 displays no effect on the translation of control mRNAs (Gray et al., 1993). This argues against the notion that IRP-1 possesses a specific binding region for eIF4G, which interferes with the binding of eIF3 to eIF4G. Earlier work showed that translation initiation is also inhibited by either of two RNA binding proteins without known functions in eukaryotic translation, the spliceosomal protein U1A and the bacteriophage MS2 coat protein, when cognate binding sites were introduced into the same location as the IRE (Stripecke and Hentze, 1992; Stripecke et al., 1994). Taking these findings into account, we suggest that the failure to recruit the small ribosomal subunit to the preassembled eIF4F/eIF4B results from steric hindrance. Such a mechanism could generally be enacted by translational regulatory proteins that bind to cap-proximal sites. The TIP assay should help to resolve this question and may prove useful in studying other examples of translational control and the translation process per se.

Experimental Procedures

Preparation of mRNA Templates

WT-IRE and ΔC-IRE mRNAs were transcribed in vitro from PvuII-linearized I-12.CAT and I-19.CAT, respectively (Gray and Hentze, 1994). The mRNA was purified from excess cap analog and unincorporated nucleotides by centrifugation through a CHROMA SPIN-100 column (Clontech). Subsequently, the mRNA was hybridized to a 5-fold molar excess of 2′O-allyl RNA oligo (complementary to nt 88–109 downstream of the CAT initiation codon) in 1× AB (100 mM KCl, 20 mM HEPES [pH 7.6]) by heating to 65°C for 5 min followed by a 30 min incubation at 37°C. To remove excess oligo, the reaction was centrifuged through a CHROMA SPIN-100 column equilibrated with 1× AB. The integrity and yield of mRNA/oligo hybrids were checked on 2% agarose or 10% polyacrylamide gels. The 2′O-allyl RNA oligo is 5′-dTbCbCbCGGUCUGGUUAUAGGUACAUUG-3′, where dN is a DNA nucleotide, N a 2′O-allyl modified RNA nucleotide, and bN a biotinylated DNA nucleotide (Lamond and Sproat, 1994).

The TIP Assay

Translation initiation intermediates were assembled from micrococcal nuclease-treated rabbit reticulocyte lysate (RRL) (Promega, WI). Sixty-microliter reactions containing 70% of RRL were incubated at 30°C for 10 min with 0.5 mM cycloheximide (Sigma) alone or, where indicated, with 2 mM GMP-PNP (Boehringer Mannheim), 0.5 mM ^{7}mGpppG (NEB), or 375 ng recombinant human IRP-1 (Gray et al., 1993). When WT-IRE mRNA was translated in the absence of IRP-1, 45 ng of IRE competitor transcripts (Gray et al., 1993) was added to the reaction to sequester IRPs endogenous to the RRL. Depending on the batch of RRL, 2 μg purified GST-PTB (Kaminski et al., 1995) was added to inhibit nonspecific ribosome binding to the mRNA (Svitkin et al., 1996). The presence or absence of GST-PTB did not affect eIF4F binding (data not shown). The initiation assays were programmed with 15–30 ng of capped WT-IRE or ΔC-IRE mRNAs, incubated for 10 min at 30°C, and placed on ice immediately afterward. Thirty microliters of streptavidin methacrylate bead suspension (Boehringer Mannheim) per reaction was prewashed three times in 3 vol of 1× WB (20 mM HEPES [pH 7.6], 100 mM KOAc, 5 mM $MgCl_2$, 80 μg/ml BSA [Sigma], 40 μg/ml poly IUC [Sigma]) and added to the initiation assay in a 100 μl volume. The mixture was tumbled gently at 4°C for 30 min, and the beads were pelleted at 2000 rpm in a microcentrifuge. The streptavidin beads were washed three times with 300 μl 1× WB. For RNA analysis, the pellet was incubated with proteinase K (100 μg) in 1× PK buffer (10 mM HEPES [pH 7.5], 0.5% SDS, 1 mM EDTA) for 15 min at 56°C and subsequently extracted once with phenol/chloroform, followed by ethanol precipitation. For protein analysis, bound translation initiation factors from six identically treated samples were pooled, and bound proteins eluted by boiling the beads in electrophoresis sample buffer (125 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.7 M 2-mercaptoethanol, 0.05% bromophenol blue) for 5 min. 18S and 28S rRNA were detected by probing with 5′ ^{32}P-labeled oligodeoxyribonucleotides (for 18S rRNA complementary to nt 432–481, for 28S rRNA to nt 1747–1797). Eluted proteins were analyzed by Western blotting and ECL detection (Amersham) with monoclonal anti-eIF4A (a generous gift from H. Trachsel; Edery et al., 1983) used in a 1:20 dilution, polyclonal anti-eIF4B (human) and anti-eIF3 (rabbit) (kind gifts from J. Hershey) used in a 1:200 and 1:500 dilution, respectively, and polyclonal antipeptide antibodies directed against the N terminus of eIF4G (generously provided by S. Morley; Morley and Pain, 1995) used in a 1:250 dilution. Rabbits were immunized with recombinant murine eIF4E expressed from pET-4E (a kind gift from N. Sonenberg; Smith et al., 1991), IgG enriched using protein A Sepharose, and used in a 1:200 dilution. Anti-IRP-1 (Pantopoulos et al., 1995) was used in a 1:200 dilution. Human autoimmune antiserum against the ribosomal P0 protein (Immunovision) was used in a 1:500 dilution. Sucrose gradient experiments were performed exactly as described (Gray and Hentze, 1994).

Acknowledgments

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References

Altmann, M., Muller, P.P., Wittmer, B., Ruchti, F., Lanker, S., and Trachsel, H. (1993). A *Saccharomyces cerevisiae* homologue of mammalian translation initiation factor 4B contributes to RNA helicase activity. EMBO J. *12*, 3997–4003.

Anthony, D.D., and Merrick, W.C. (1992). Analysis of 40 S and 80 S complexes with mRNA as measured by sucrose density gradients and primer extension inhibition. J. Biol. Chem. *267*, 1554–1562.

Asano, K., Vornlocher, H.P., Richter-Cook, N.J., Merrick, W.C., Hinnebusch, A.G., and Hershey, J.W. (1997). Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. J. Biol. Chem. *272*, 27042–27052.

Beaumont, C., Leneuve, P., Devaux, I., Scoazec, J., Berthier, M., Loiseau, M., Grandchamp, B., and Bonneau, D. (1995). Mutation in the iron responsive element of the L ferritin mRNA in a family with dominant hyperferritinaemia and cataract. Nat. Genet. *11*, 444–446.

Beelman, C.A., and Parker, R. (1995). Degradation of mRNA in eukaryotes. Cell *81*, 179–183.

Britton, R.S., Tavill, A.S., and Bacon, B.R. (1994). Mechanisms of

iron toxicity. In Iron Metabolism in Health and Disease, J.H. Brock, J.W. Halliday, M.J. Pippard, and L.W. Powell, eds. (London: W.B. Saunders), 311–351.

Edery, I., Humbelin, M., Darveau, A., Lee, K.A., Milburn, S., Hershey, J.W., Trachsel, H., and Sonenberg, N. (1983). Involvement of eukaryotic initiation factor 4A in the cap recognition process. J. Biol. Chem. *258*, 11398–11403.

Girelli, D., Corrocher, R., Bisceglia, L., Olivieri, O., De Franceschi, L., Zelante, L., and Gasparini, P. (1995). Molecular basis for the recently described hereditary hyperferritinemia-cataract syndrome: a mutation in the iron-responsive element of ferritin L-subunit gene. Blood *86*, 4050–4053.

Goossen, B., and Hentze, M.W. (1992). Position is the critical determinant for function of iron-responsive elements as translational regulators. Mol. Cell. Biol. *12*, 1959–1966.

Gray, N.K., and Hentze, M.W. (1994). Iron regulatory protein prevents binding of the 43S translation pre-initiation complex to ferritin and eALAS mRNAs. EMBO J. *13*, 3882–3891.

Gray, N.K., Quick, S., Goossen, B., Constable, A., Hirling, H., Kühn, L.C., and Hentze, M.W. (1993). Recombinant iron regulatory factor functions as an iron-responsive element-binding protein, a translational repressor and an aconitase. A functional assay for translational repression and direct demonstration of the iron switch. Eur. J. Biochem. *218*, 657–667.

Haghighat, A., and Sonenberg, N. (1997). eIF4G dramatically enhances the binding of eIF4E to the mRNA 5′-Cap structure. J. Biol. Chem. *272*, 21677–21680.

Halliday, J.W., Ramm, G.A., and Powell, L.W. (1994). Cellular iron processing and storage: the role of ferritin. In Iron Metabolism in Health and Disease, J.H. Brock, J.W. Halliday, M.J. Pippard, and L.W. Powell, eds. (London: W.B. Saunders), 97–122.

Hentze, M.W. (1997). eIF4G: a multipurpose ribosome adapter? Science *275*, 500–501.

Hentze, M.W., and Kühn, L.C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. Proc. Natl. Acad. Sci. USA *93*, 8175–8182.

Hershey, J.W. (1991). Translational control in mammalian cells. Annu. Rev. Biochem. *60*, 717–755.

Hershey, J.W., and Monro, R.E. (1966). A competitive inhibitor of the GTP reaction in protein synthesis. J. Mol. Biol. *18*, 68–76.

Imataka, H., and Sonenberg, N. (1997). Human eukaryotic translation initiation factor 4G (eIF4G) possesses two independent binding sites for eIF4A. Mol. Cell. Biol. *17*, 6940–6947.

Joshi, B., Yan, R., and Rhoads, R.E. (1994). *In vitro* synthesis of human protein synthesis initiation factor 4γ and its localization on 43 and 48 S initiation complexes. J. Biol. Chem. *269*, 2048–2055.

Kaminski, A., Hunt, S.L., Patton, J.G., and Jackson, R.J. (1995). Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. RNA *1*, 924–938.

Lamond, A.I., and Sproat, B. (1994). Isolation and characterization of ribonucleoprotein complexes. In RNA Processing—A Practical approach, S.J. Higgins and B.D. Hames, eds. (Oxford: Oxford University Press), pp. 103–140.

Lamphear, B.J., Kirchweger, R., Skern, T., and Rhoads, R.E. (1995). Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. J. Biol. Chem. *270*, 21975–21983.

Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. Mol. Cell. Biol. *15*, 4990–4997.

Merrick, W.C. (1992). Mechanism and regulation of eukaryotic protein synthesis. Microbiol. Rev. *56*, 291–315.

Methot, N., Song, M.S., and Sonenberg, N. (1996). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol. Cell. Biol. *16*, 5328–5334.

Morley, S.J., and Pain, V.M. (1995). Hormone-induced meiotic maturation in *Xenopus* oocytes occurs independently of p70s6k activation and is associated with enhanced initiation factor (eIF)-4F phosphorylation and complex formation. J. Cell Sci. *108*, 1751–1760.

Morley, S.J., Curtis, P.S., and Pain, V.M. (1997). eIF4G: translation's mystery factor begins to yield its secrets. RNA *10*, 1085–1104.

Obrig, T.G., Culp, W.J., McKeehan, W.L., and Hardesty, B. (1971). The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J. Biol. Chem. *246*, 174–181.

Ohlmann, T., Rau, M., Morley, S.J., and Pain, V.M. (1995). Proteolytic cleavage of initiation factor eIF-4γ in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. Nucleic Acids Res. *23*, 334–340.

Pain, V.M. (1996). Initiation of protein synthesis in eukaryotic cells. Eur. J. Biochem. *236*, 747–771.

Pantopoulos, K., Gray, N.K., and Hentze, M.W. (1995). Differential regulation of two related RNA-binding proteins, iron regulatory protein (IRP) and IRP_B. RNA *1*, 155–163.

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. EMBO J. *11*, 2643–2654.

Rau, M., Ohlmann, T., Morley, S.J., and Pain, V.M. (1996). A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. J. Biol. Chem. *271*, 8983–8990.

Rhoads, R.E., Joshi, B., and Minich, W.B. (1994). Participation of initiation factors in the recruitment of mRNA to ribosomes. Biochimie *76*, 831–838.

Rouault, T.A., Hentze, M.W., Caughman, S.W., Harford, J.B., and Klausner, R.D. (1988). Binding of a cytosolic protein to the iron-responsive element of human ferritin messenger RNA. Science *241*, 1207–1210.

Rouault, T., Klausner, R.D., and Harford, J.B. (1996). Translational control of ferritin. In Translational Control, J.W.B. Hershey, M.B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 335–362.

Rozen, F., Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C., and Sonenberg, N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol. Cell. Biol. *10*, 1134–1144.

Sachs, A.B., Sarnow, P., and Hentze, M.W. (1997). Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell *89*, 831–838.

Smith, M.R., Jaramillo, M., Tuazon, P.T., Traugh, J.A., Liu, Y.L., Sonenberg, N., and Kung, H.F. (1991). Modulation of the mitogenic activity of eukaryotic translation initiation factor-4E by protein kinase C. New Biol. *3*, 601–607.

Stripecke, R., and Hentze, M.W. (1992). Bacteriophage and spliceosomal proteins function as position-dependent *cis/trans* repressors of mRNA translation in vitro. Nucleic Acids Res. *20*, 5555–5564.

Stripecke, R., Oliveira, C.C., McCarthy, J.E., and Hentze, M.W. (1994). Proteins binding to 5′ untranslated region sites: a general mechanism for translational regulation of mRNAs in human and yeast cells. Mol. Cell. Biol. *14*, 5898–5909.

Svitkin, Y.V., Ovchinnikov, L.P., Dreyfuss, G., and Sonenberg, N. (1996). General RNA binding proteins render translation cap dependent. EMBO J. *15*, 7147–7155.