Viral Stress-inducible Protein p56 Inhibits Translation by Blocking the Interaction of eIF3 with the Ternary Complex eIF2-GTP-Met-tRNA\textsubscript{i}*

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Viral stress-inducible protein p56 is produced in response to viral stress-inducing agents such as double-stranded RNA and interferon, as well as other poorly understood mechanisms of viral infection. It has been shown previously that p56 is able to bind the eukaryotic initiation factor 3e (eIF3e) (p48/Int-6) subunit of the eukaryotic translation initiation factor eIF3 and function as an inhibitor of translation \textit{in vitro} and \textit{in vivo}. The exact mechanism by which p56 is able to interfere with protein synthesis is not understood. Based on the known roles of eIF3 in the initiation pathway, we employed assays designed to individually look at specific functions of eIF3 and the effect of p56 on these eIF3-mediated functions. These assays examined the effect of p56 on ribosome dissociation, the eIF3-eIF4F interaction, and enhancement of the ternary complex eIF2-GTP-Met-tRNA\textsubscript{i}, formation. Here we report that p56 is able to inhibit translation initiation specifically at the level of eIF3-ternary complex formation. The effect of p56-mediated inhibition was also examined in two different contexts, cap-mediated and encephalomyocarditis virus internal ribosomal entry site-mediated translation. Whereas cap-dependent initiation was severely inhibited by p56, internal ribosomal entry site-mediated translation appeared to be insensitive to p56.

Viral stress-inducible protein p56 is a 56-kDa protein that is induced by various agents characteristic of viral infection, such as double-stranded RNA (dsRNA), interferon, and by other less well understood mechanisms associated with viral infection (1). Previous work has characterized the transcriptional regulation of the p56 gene, also known as the 561 gene, IG556, and IFIT1 (2), as well as the cellular function of the protein \textit{in vitro} and \textit{in vivo} (3). p56 belongs to a family of related viral stress-inducible proteins that includes p54, p56, p58, and p60 (4–8), but the other members of the family are not as well characterized nor are their cellular functions known. Structurally, the only significant feature of p56 is the presence of eight tetratricopeptide (TPR) motifs, motifs shown in other proteins to mediate protein-protein interactions (9). One of the best characterized interactions with p56 identified thus far has been with Int-6/p48 (also known as eIF3e), a subunit of eukaryotic initiation factor 3 (eIF3) (10). Binding of p56 to the eIF3e subunit has been shown to have a functional effect by inhibiting overall cellular translation (3). Whereas the functional effect of p56 has been mapped to TPR motifs 6–8, the mechanism by which p56 inhibits translation is not known (10).

eIF3 is one of the 11 or more initiation factors that are involved in the first stage of protein synthesis in eukaryotes. Mammalian eIF3 is the largest (650 kDa) of all the initiation factors and is composed of 11 or 12 individual subunits eIF4-eIF1 (11). The exact interactions and stoichiometry of the eIF3 subunits are poorly understood. eIF3 is a multifunctional initiation factor that has been shown to operate at different levels of the initiation pathway. eIF3 serves as a dissociation factor by binding to the 40 S ribosomal subunit, thereby preventing re-association with the 60 S subunit and thereby increasing the size of the 40 S subunit pool (12, 13). eIF3 also functions by interacting with eIF2 and stabilizing the interaction between the ternary complex (composed of eIF2-GTP-Met-tRNA\textsubscript{i}), and the 40 S ribosomal subunit, thereby forming the 43 S ribosomal complex (14, 15). Finally, eIF3 has been shown to interact with initiation factor eIF4G of the heterotrimeric eIF4F complex, which also consists of eIF4A and eIF4E (16). The interaction between eIF3 and eIF4F mRNA serves to bring the mRNA to the 43 S ribosomal complex, thereby forming the 48 S complex (17).

eIF3 is also known to play a role in alternative, cap-independent mechanisms of translation that are still being elucidated. Many viral, as well as cellular, mRNAs have evolved internal ribosomal entry sites (IRES) to provide a site for ribosome attachment. Although the initiation factor requirements for translation appear to be different depending on the IRES, eIF3 has already been shown to be required for encephalomyocarditis virus (EMCV) and hepatitis C virus (HCV) IRES-mediated translation (18, 19). Thus, eIF3 provides a possible site of regulation by p56 at both the cap-dependent and cap-independent levels.

Based on the known functions of eIF3, the role of p56 and the mechanism of inhibition were examined further by using inhibition of eIF3-function assays. It was found that p56 inhibits translation specifically at the level of eIF3-ternary complex formation. Furthermore, the inhibition appears to act preferentially on cap-mediated translation, whereas EMCV IRES-mediated translation was apparently insensitive to inhibition.
The addition of exogenous eIF2 stimulated cap-mediated translation, but not 48S-mediated translation, suggesting that excess levels of eIF2 (and thereby ternary complex) may be able to compete with p56 for binding to either free eIF3 or 40S bound eIF3.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal antibody against p56 was obtained by injection of purified p56 protein expressed in Esherichia coli as described previously (3). A polyclonal antibody raised in goat against purified rabbit eIF3 was also used in these studies. This antibody recognized primarily the p110 (eIF3c) subunit of eIF3 and other eIF3 subunits to a lesser extent.

Purification of Recombinant p56 from E. coli—p56 and mutant p56 were purified as described previously (3). Briefly, full-length or mutant cDNA lacking TPR region 6–8 was cloned into the pET15b vector (Novagen), expressed in E. coli, and purified via nickel affinity chromatography. Protein was then dialyzed against a high glycerol buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 0.5 mM diethiothreitol (DTT), 0.5 mM EDTA, 50% glycerol) and stored at -20 °C.

Initiation Factors—eIF2, eIF3, and eIF4F were purified from rabbit reticulocyte lysate as described previously (20, 21).

Generation of Radiolabeled eIF4F—Radiolabeled eIF4F was generated in vitro via reductive methylation using [3H]formaldehyde (PerkinElmer Life Sciences) as described previously (22).

Purification of Ribosomal Subunits—Free 40S and 60S ribosomal subunits were purified using high salt sucrose gradients as described previously (13, 20).

Generation of [14C]Met-tRNAi—Radiolabeled Met-tRNAi, was prepared using Brewer’s yeast tRNA (Ambion), E. coli aminoaacyl-tRNA synthetase, and [3H]methionine (56 mCi/mmol, PerkinElmer Life Sciences) as described previously (20).

Ribosome Dissociation Assay—The ribosome dissociation assay was performed as described in Ref. 13. Purified 40S ribosomal subunits (0.7 A260 units) were incubated with 1.4 A260 units purified 60S ribosomal subunits to form 80S ribosomes in a 100-μl reaction volume containing 100 mM KCl, 10 mM Tris–HCl, pH 7.5, 3 mM MgCl2, and 2 mM DTT. To dissociate ribosomes, 60 pmol of eIF3 (37.5 μg, 600 nM) was added to the reaction, incubated for 10 min at 37 °C, then layered on a 12–20–25% sucrose gradient (100 mM KCl, 20 mM Hepes-KOH, pH 7.5, 5 mM MgCl2, 2 mM DTT), and centrifuged for 16 h at 20,000 rpm at 4 °C (Beckman SW40Ti rotor). To test the effect of p56, 60 pmol of purified p56 (3.2 μg) and mutant p56 was pre-incubated with eIF3 for 10 min at 30 °C prior to their addition to the ternary complex reaction mixture.

Generation of a Bicistronic Reporter mRNA—A bicistronic plasmid, containing the EMCV IRES (420 nucleotides) intervening between the chloramphenicol acetyltransferase (CAT, ORF1) and firefly luciferase (LUC, ORF2) reporter genes and designated pGEM-CAT-EMCV-LUC (24), was a generous gift from Dr. Nahum Sonenberg, McGill University, Montreal, Canada. The bicistronic (CAT-EMCV-LUC) insert was introduced downstream of the viral T7 RNA polymerase promoter and contains a unique restriction site, Xhol, that was used to linearize the plasmid prior to transcription. In vitro transcription was carried out for 2 h at 37 °C in the presence of m7G(“)ppp(5”)G using Megascript transcription kit and procedures outlined by Ambion. Following the transcription reaction, samples were treated with RNase-free DNase for 10 min and phenol/chloroform/isoamyl alcohol extracted twice, ethanol precipitated, and washed in 70% ethanol. RNA samples were recovered in water and quantitated using a Hewlett-Packard Chemstation 8453 spectrophotometer and monitored for integrity by ethidium bromide staining following electrophoresis on acrylamide/urea gels.

In Vitro Translation Inhibition Assay—In vitro translations in rabbit reticulocyte lysate were performed with the capped CAT-EMCV-LUC transcript in the presence of [35S]methionine. In vitro translations were performed with nuclease-treated rabbit reticulocyte lysate under conditions recommended by Promega. A typical 15-μl reaction contained 10 μl of lysate, 0.2 μM amino acid mixture (minus methionine), 6 μCi of [35S]methionine (1200 Ci/mmol), 24 units of RNase inhibitor (Roche Applied Science), 0.4 μg of transcript (containing 25 μg/ml) of purified eIF2 was added in increasing amounts as indicated in the figure legends (20, 21, 23). Translations were performed at 30 °C for 1 h in the presence of increasing amounts of wild type or mutant p56. Following translation, an aliquot of the lysate was resolved by 12.5% SDS-PAGE, and gels were dried and exposed to a PhosphorImager screen, and incorporated radioactivity was quantitated using the ImageQuant software from Amersham Biosciences.

RESULTS

p56 Does Not Interfere with eIF3-mediated Ribosome Dissociation—Because eIF3 is an essential initiation factor whose function in the initiation of translation is implicated at the earliest step of the initiation pathway, studies with p56 began at the level of ribosome dissociation. Fig. 1 shows the results of a size-separation assay by using sucrose gradient centrifugation to analyze the dissociation of ribosomes by eIF3. In this assay, individual preparations of purified 40S and 60S ribosomal units were used rather than salt-washed ribosomes, because they produced clearer UV profiles, separating into distinct peaks (data not shown). The UV profile of the gradient remained unchanged in the presence of p56 protein buffer as

**Fig. 1.** p56 does not inhibit eIF3-mediated enhancement of ribosome dissociation. UV absorbance profiles of 10–25% sucrose gradients are shown with increasing density from left to right. A, purified 40S ribosomal subunits (0.7 A260 units) were incubated with 1.4 A260 purified 60S ribosomal subunits to form 80S ribosomes in the presence of eIF3 buffer as a control. B, same as in A with eIF3 (60 pmol, 37.5 μg) added; C same as in B with equimolar (600 nm, 60 pmol, 3.2 μg) p56 pre-incubated with eIF3.
p56 Inhibits eIF3-Ternary Complex

Fig. 2. p56 association with 40 S ribosomal subunits is eIF3-dependent. A, an A260 UV profile of 40 S ribosomal subunits on a 10–25% sucrose gradient is shown. The 40 S peak corresponds to fraction 8 as shown from increasing density going left to right. B, p56 Western blot of fractions from sucrose gradient analysis of a reaction mixture containing 0.7 A260 40 S subunits and 60 pmol (600 nM, 3.2 µg) of p56 only. C, p56 Western blot of fractions from sucrose gradient analysis of a reaction mixture containing 0.7 A260 40 S subunits, 3.2 µg of p56 in addition to 60 pmol (600 nM, 37.5 µg) of eIF3.

shown in Fig. 1A. Because eIF3 serves as an anti-association factor, addition of eIF3 to the reaction would be expected to "dissociate" 80 S ribosomes by binding to the small pool of 40 S ribosomal subunits and prevent reassociation with 60 S subunits, effectively shifting the equilibrium to free 40 S subunits.

In Fig. 1B, 60 pmol of eIF3 in an approximate 2:1 molar excess of ribosomes resulted in a dramatic decrease in the 80 S peak and a corresponding increase in the 40 S peak, which suggests an increase in the amount of 60 S formed. We did not observe an equivalent increase in the 40 S ribosomal subunit pool. In part, this appears to reflect a tendency of mammalian 40 S subunits to dimerize (see Fig. 4 of Ref. 25), and this peak did not resolve well from the peak of 60 S subunits.

To investigate the possibility of p56 interfering with the function of eIF3 as a dissociation factor, an equimolar amount of p56 protein was pre-incubated with eIF3 prior to the addition of the ribosomal subunits. After sucrose gradient centrifugation, the activity of eIF3 as a dissociation factor was unaffected, as the peak corresponding to 80 S subunits was still reduced, and the peaks corresponding to the 40 S and 60 S subunit pool increased (Fig. 1C). As expected, a mutant form of p56, lacking the interaction domain (TPR6–8) (10), did not affect the dissociation of ribosomes, producing a profile similar to only eIF3 added (data not shown).

p56 Does Not Interfere with the eIF3-eIF4F Interaction—eIF3 is also known to play an important role in binding and stabilization of the eIF4F complex on the 40 S subunit. The eIF4F complex is responsible for binding and unwinding the mRNA prior to translation. Because p56 was shown not to interfere with the ability of eIF3 to dissociate ribosomes, inhibition could be mediated through the disruption of the eIF3-eIF4F interaction. To examine the effect of p56 on the eIF3-eIF4F interaction, a sucrose gradient separation assay was performed using radiolabeled eIF4F (200 kDa) and unlabeled eIF3 (650 kDa). As shown in Fig. 4A, 150 pmol of radiolabeled eIF4F remained in the upper region of a 5–18% sucrose gradient, as measured by liquid scintillation spectrometry of sucrose gradient fractions. When equimolar (150 pmol) eIF3 was incubated in the presence of eIF4F, a shift in radioactivity was observed from the upper region of the gradient, toward the middle of the gradient, corresponding to fractions containing eIF3 (Fig. 4B). The shift in radioactivity represents the formation of the complex of eIF3 and eIF4F. The efficiency of eIF4F binding to eIF3 was ~50%, which is consistent with previous findings (22) reporting the eIF3-eIF4F interaction.

conditions, the 40 S ribosomes migrated in fraction 8. When both 60 pmol of p56 and 60 pmol of eIF3 were added to the reaction containing ~20 pmol of 40 S ribosomal subunits, p56 was able to bind to eIF3, which formed a complex with the 40 S subunit, as shown by Western blot analysis of gradient fractions using an antibody to p56 (Fig. 2C). p56 migration with the 40 S ribosomal subunit was dependent on the interaction with eIF3, as p56 incubated alone with 40 S subunits did not bind and remained in the upper region of the gradient (Fig. 2B). Similarly, eIF3 alone remained at the top of the gradient in the absence of 40 S ribosomal subunits, as visualized by Coomassie staining of sucrose gradient fractions (data not shown).

To confirm that p56 was not interfering with the ability of eIF3 to bind to 40 S ribosomal subunits, a Western blot analysis was performed using a polyclonal antibody against eIF3. As seen in Fig. 3A, this antibody was able to recognize primarily the p110 subunit of eIF3 (eIF3c), but at longer exposure times other subunits could also be detected, although to a lesser degree. When fractions from a sucrose gradient containing 0.7 A260 units of 40 S ribosomal subunits and 60 pmol of eIF3 (at an ~3-fold molar excess to ribosomal subunits) were subjected to Western blot analysis, eIF3 could be seen migrating in the fractions corresponding to 40 S subunits (Fig. 3B). This association remained unaffected, even in the presence of equimolar p56 (Fig. 3C), indicating that p56 had no effect on the ability of eIF3 to bind to 40 S subunits.

p56 Does Not Interfere with the eIF3-eIF4F Interaction—eIF3 is also known to play an important role in binding and stabilization of the eIF4F complex on the 40 S subunit. The eIF4F complex is responsible for binding and unwinding the mRNA prior to translation. Because p56 was shown not to interfere with the ability of eIF3 to dissociate ribosomes, inhibition could be mediated through the disruption of the eIF3-eIF4F interaction. To examine the effect of p56 on the eIF3-eIF4F interaction, a sucrose gradient separation assay was performed using radiolabeled eIF4F (200 kDa) and unlabeled eIF3 (650 kDa). As shown in Fig. 4A, 150 pmol of radiolabeled eIF4F remained in the upper region of a 5–18% sucrose gradient, as measured by liquid scintillation spectrometry of sucrose gradient fractions. When equimolar (150 pmol) eIF3 was incubated in the presence of eIF4F, a shift in radioactivity was observed from the upper region of the gradient, toward the middle of the gradient, corresponding to fractions containing eIF3 (Fig. 4B). The shift in radioactivity represents the formation of the complex of eIF3 and eIF4F. The efficiency of eIF4F binding to eIF3 was ~50%, which is consistent with previous findings (22) reporting the eIF3-eIF4F interaction.
p56 Inhibits eIF3-Ternary Complex

To test the effect of p56 on the formation of the eIF3-eIF4F complex, an equimolar (150 pmol) amount of purified p56 was pre-incubated with eIF3 prior to incubation with eIF4F. After separation on the gradient, the radioactivity profile remained unchanged, showing that eIF4F was still able to form a complex with eIF3, even in the presence of p56 (Fig. 4C). To confirm that p56 was present in the eIF3-eIF4F complex, Western blot analysis of sucrose gradient fractions with the p56 antibody demonstrated the presence of p56 in the fractions containing eIF3 (data not shown). As expected, the mutant form of p56 did not change the radioactivity profile of the eIF4F (Fig. 4D). Therefore, the eIF3-eIF4F interaction is unaffected by p56 and is unlikely to be responsible for the p56-mediated inhibition of protein synthesis.

p56 Inhibits the Enhancement of Ternary Complex Formation by eIF3—Another step of the initiation pathway that eIF3 is known to operate at, and thus representing a possible target site for p56 inhibition, is the formation and stabilization of the ternary complex. eIF3 binds the ternary complex, which consists of eIF2, initiator Met-tRNAi, and GTP, and in doing so shifts the equilibrium toward ternary complex and ternary complex-eIF3 (15). To investigate the possible effect of p56 on ternary complex formation, a filter binding assay was employed. This assay is based on the principle that Met-tRNAi binding to a nitrocellulose filter is dependent on the binding to an initiation factor (26). Thus, in the presence of eIF2, which is retained on nitrocellulose, Met-tRNAi will be retained on the filter by binding to eIF2 in the presence of GTP. By using radiolabeled Met-tRNAi, formation of the ternary complex can be quantitatively measured.

Presented in Fig. 5 are the cumulative results of 5 separate filter-binding assays as described under “Materials and Methods.” As expected, [14C]Met-tRNAa was not retained on the nitrocellulose filter, and all other values were normalized to the background levels of radioactivity detected (Fig. 5, column A). In the presence of 8 pmol (1 μg) of eIF2 and GTP, [14C]Met-tRNAa was retained on the filter, representing ternary complex formation (Fig. 5, column B). The addition of 30 pmol of eIF3 to the reaction resulted in a 4-fold increase in radioactivity retained on the nitrocellulose filter (Fig. 5, column C), representing the effect of eIF3 on the stabilization of ternary complex formed. Of particular interest, when 30 pmol of p56 was pre-incubated with equimolar eIF3, the result was a complete abrogation of the eIF3 stimulatory effect on ternary complex formation (Fig. 5, column E), providing the first evidence of a possible mechanism of inhibition. Additional evidence supporting this observation was the relative inability of mutant p56 to inhibit the eIF3-mediated stimulation of ternary complex (Fig. 5, column F), presumably due to its inability to bind to eIF3 (10).

p56 Exhibits Differential Effects on m7G Cap and IRES-mediated Translation Initiation—Once the specific stage of the initiation pathway at which p56 inhibited translation had been implicated, p56 was investigated for its ability to inhibit in vitro translation in two different contexts, cap-mediated and IRES-mediated translation initiation. The reporter used was a bicistronic mRNA, containing the EMCV IRES. The EMCV IRES requires all canonical initiation factors for translation to initiate internally (19). Because ternary complex is required for both cap-mediated and IRES-mediated translation, it would seem likely that both modes of translation would be equally inhibited by p56.

Surprisingly, p56 did not appear to inhibit IRES-mediated translation at the levels of p56 added. Translation of the cap-dependent open reading frame, CAT was inhibited in a dose-dependent manner (Fig. 6A, lanes 1–6), which is consistent with our findings reported previously (3). However, translation of the IRES-dependent open reading frame, luciferase (LUC), was not inhibited upon addition of p56, suggesting that the EMCV IRES has an alternative mechanism of translation that is less sensitive to levels of ternary complex. Addition of exogenous eIF2 was able to partially restore cap-dependent translation (Fig. 6A, lane 7). Quantitation of the two products is shown in Fig. 6B, demonstrating the dose-dependent inhibitory effect on CAT translation by increasing amounts of p56, whereas LUC translation remained unaffected. Consequently, the ratio of CAT translation to LUC translation decreased (Fig. 6C). In a similar experiment, mutant p56 did not inhibit either cap- or IRES-mediated translation, even when tested at higher concentrations (560 nM; data not shown).

DISCUSSION

Previous efforts identified that p56 was capable of binding the p48 (Int-6) subunit of eIF3 and that this binding could lead to the inhibition of protein synthesis, although the mechanism of inhibition was not defined (3). eIF3 is a large, multisubunit complex and appears to coordinate a number of events in the 80 S initiation pathway (11). The best characterized of these interactions is...
Fig. 6. p56 selectively inhibits cap-dependent but not IRES-mediated translation. A, bicistronic construct containing the EMCV IRES, intervening between the CAT and LUC open reading frames was employed to generate capped in vitro transcripts. Rabbit reticulocyte lysate was programmed with the CAT-EMCV-LUC transcripts in the presence of [35S]methionine. Increasing amounts of purified p56 was added to the lysate, and translations were performed for 1 h at 30°C. The lanes correspond as follows: no p56 (lane 1); 70 nM p56 (lane 2); 140 nM p56 (lane 3); 280 nM p56 (lane 4); 420 nM p56 (lane 5); 560 nM p56 (lane 6); 280 nM p56 and 800 nM eIF2 (1.5 µg) (lane 7). The bands corresponding to CAT (ORF1) and LUC (ORF2) translation are indicated with an arrow, as is the position of the β-14C-galactosidase, which was introduced into the lysate to correct for loading variation. The relative position of the molecular mass markers used (14C-radiolabeled Rainbow markers, Amersham Biosciences) is indicated on the left side and corresponds to 97.0, 68.0, 45.0, 30.0, and 20.1 kDa. The values shown in B are aligned to their respective lanes and were calculated from quantitations of radioactivity using a PhosphorImager. The values are expressed relative to counts obtained for CAT (ORF1, 100%) with no p56. Calculations were normalized to radioactivity obtained for β-14C-galactosidase in respective lanes. C represents the ratio (CAT/LUC) obtained for in vitro translation in the presence of increasing amounts of p56.

the ability of eIF3 to bind to 40 S subunits, eIF4F, and the ternary complex (11, 15, 21). We have systematically examined the ability of p56 to interfere with each of these processes and observed that only the third interaction was affected.

It has been shown that under conditions of limiting Met-tRNAi, eIF3 enhances retention of Met-tRNAi on nitrocellulose filters (15) presumably by forming an eIF3-ternary complex and thus pulling the following equilibrium (Reaction 1) to the right:

\[
\text{Met-tRNA_i} + \text{eIF2-GTP} \rightleftharpoons \text{eIF2-GTP-Met-tRNA_i} \rightleftharpoons \text{eIF3-eIF2-GTP-Met-tRNA_i} \rightarrow \text{ternary complex}
\]

**REACTION 1**

At present it is not clear whether this interaction reflects a sequence whereby eIF3 binds to 40 S subunits as an eIF3-ternary complex or whether this reflects the intimate interaction of these proteins on the surface of the 40 S subunit. As seen in Fig. 5, p56 completely inhibited this interaction. The consequence of limiting the eIF3-ternary complex interaction is depicted in Fig. 7. Arrows labeled A and B show the possible sites where p56 might have its effect, either by blocking the binding of the ternary complex to the 40 S-eIF3 complex (A) or by blocking the direct interaction between eIF3 and the ternary complex (B). Either of these steps would lead to reduced levels of 40 S-eIF3-ternary complexes and a general overall slowing of protein synthesis initiation.

This study demonstrates the first example of differential regulation of the three functions of eIF3 in translation initiation. Although binding of p56 inhibited the interaction of eIF3 with the ternary complex, its other two interactions (either with 40 S subunits or eIF4F) remained unaffected. This observation suggests that different domains of eIF3 interface with different interacting partners, and these domains can function independently of one another. It remains to be seen if there are other cellular proteins that can bind to other subunits of eIF3 and regulate the functions unaffected by p56. Independent regulation of the different activities of eIF3 may be needed for selective, but not global, regulation of translation initiation as observed for the effect of p56 on translation of the bicistronic mRNA. eIF3-interacting proteins, such as p56, may be useful tools for mapping the functional topology of this multiprotein complex.

As a further test of p56 action, p56 was tested for its ability to inhibit the translation of the bicistronic mRNA, p6EM-CAT-EMCV IRES-LUC. Surprisingly, there appeared to be little or no inhibition of the IRES-mediated translation of LUC. This observation is consistent with preliminary studies using hemodficient lysate or normal lysate supplemented with dsRNA. Both of these translation systems contain reduced levels of ternary complex, relative to either normal lysate or lysate
supplemented with exogenous eIF2, and only inhibition of the cap-dependent translation was observed.

Does this preferential inhibition of cap-dependent protein synthesis make sense? The answer would appear to be yes. First, the preferential inhibition of cap-dependent translation appears to be exactly the same as that observed with PKR, an interferon induced eIF2alpha kinase that is activated by dsRNA, the proverbial “calling card” of viral infection. Second, interferon action is not exclusively restricted to a protective effect against viral infections. Interferons have been shown to have a broad range of biologically protective functions. In this light, it should be noted that many, but not all, proteins associated with response to stressing events contain IRES elements (27). Although it is not clear that these cellular IRESs all behave identically to the EMVC IRES, it could be imagined that many would. Thus, these IRES containing mRNAs would be resistant to the general reduction in ternary complexes that could have resulted from the action of any of the four eIF2alpha kinases (HRI, PKR, PERK, and GCN2).

In addition, many stressing agents also influence eIF4F activity, primarily through the eIF4E/4E-BP interaction (27). The reduction of eIF4F activity under stress directly down-regulates cap-dependent translation as expected and simultaneously allows for the up-regulation of IRES-mediated translation (28). In sum, the ability to maintain IRES-mediated translation for the expression of a variety of protective proteins in the presence of stressing agents or in response to interferon stimulation, which may influence ternary complex levels and/or eIF4F activity, ensures optimal protection for the cell.

The unanswered question is why is IRES-mediated translation less sensitive to ternary complex levels? The general 80 S initiation pathway would predict that both cap-dependent and IRES-mediated translation initiation should be reduced when the level of 40 S ternary complexes fall (43 S complexes) and thereby form a more effective trap for the 40 S ribosome. Finally, the IRES element bind directly to the 40 S ribosome. It will also be of interest to see if the effectiveness of interferon treatment in vivo is generally better against viruses that generate mRNAs that are translated in a cap-dependent manner than against viruses that rely on IRES-mediated translation of their mRNAs.

It is curious to note that the lack of inhibitory effects of p56 on EMCV IRES-mediated translation observed in the current study may not be universally applicable to all IRES elements in all cell types. Wang et al. (35) reported that in HuH7 cells, p56 could strongly inhibit translation directed by the HCV IRES. This inhibition was less pronounced for both cap-dependent and EMCV IRES-dependent translation. The higher sensitivity of HCV IRES to p56, as compared with cap-dependent translation, was also observed in vitro using rabbit reticulocyte lysates (35). Taken together with the conclusions of Wang et al. (35), our study suggests a hierarchy of p56 susceptibility; EMCV IRES is much less sensitive than cap-dependent initiation, and HCV IRES is more sensitive. The underlying biochemical mechanism responsible for this hierarchy is currently unclear, although the known difference in the ways EMVC IRES and HCV-IRES recruit the 40 S subunit is expected to play an important role (36).

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