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An inhibitor of eIF2 activity in the sRNA pool of eukaryotic cells

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Abstract

Eukaryotic protein synthesis is a multi-step and highly controlled process that includes an early initiation complex containing eukaryotic initiation factor 2 (eIF2), GTP, and methionine-charged initiator methionyl-tRNA (met-tRNAi). During studies to reconstruct formation of the ternary complex containing these molecules, we detected a potent inhibitor in low molecular mass RNA (sRNA) preparations of eukaryotic tRNA. The ternary complex inhibitor (TCI) was retained in the total sRNA pool after met-tRNAi was charged by aminoacyl tRNA synthetase, co-eluted with sRNA by size exclusion chromatography, but resolved from met-tRNAi by ion exchange chromatography. The adverse effect of TCI was not overcome by high GTP or magnesium omission and was independent of GTP regeneration. Rather, TCI suppressed the rate of ternary complex formation, and disrupted protein synthesis and the accumulation of heavy polymeric ribosomes in reticulocyte lysates *in vitro*. Lastly, a component or components in ribosome depleted cell lysate significantly reversed TCI activity. Since assembly of the met-tRNAi/eIF2/GTP ternary complex is integral to protein synthesis, awareness of TCI is important to avoid confusion in studies of translation initiation. A clear definition of TCI may also allow a better appreciation of physiologic or pathologic situations factors and events that control protein synthesis *in vivo*.

Keywords

translation initiation; methionyl-tRNA; ternary complex

INTRODUCTION

Protein synthesis in eukaryotic cells is controlled by a variety of events, many related to a stress response where the net rate of translation is suppressed. This coincides with a sharp reduction in the binding of methionine-charged initiator methionyl-tRNA (met-tRNAi) to the 40S ribosomal subunit prior to its engagement with mRNA and a disaggregating effect on heavy polymeric ribosomes into 80S monomers. Many links between stress and the initiation of translation have been identified, including those that involve the formation of a pre-translation ternary complex comprising met-tRNAi, GTP, and the eukaryotic protein synthesis initiation factor termed eIF2 (Holcik and Sonenberg, 2005; Kaneto et al., 2006; Ranganathan et al., 2006; Wek et al., 2006). eIF2 is a heterotrimeric protein composed of an

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α subunit that contains a phosphorylation sensitive regulatory site at serine 51; a β subunit that binds tRNA and mRNA, and contains both a zinc finger associated with initiation and ribosomal subunit binding and a protein interaction domain for the multimeric guanine nucleotide exchange factor eIF2B; and a γ subunit that contains a zinc binding domain and an essential GTP/GDP docking site (Proud, 2005). eIF2 activity is controlled in many ways. Of these, the best studied are various forms of nutritional, cytokine, infection, or chemically induced stress which activate one of several kinases that phosphorylate the eIF2 α subunit (Hinnebusch, 1993; Olmsted et al., 1993; Sood et al., 2000; Chen, 2007; Garcia et al., 2007; Sadler and Williams, 2007; Raven and Koromilas, 2008; Zaborske et al., 2009). Phosphorylated eIF2 α binds and potently inhibits the guanine nucleotide exchange potential of eIF2B, which occurs in a far lower concentration than eIF2. Therefore phosphorylation of even a small fraction of total eIF2 α can rapidly block the release of GDP from eIF2 and the ability of eIF2 to recycle through the processes of ternary complex formation and protein synthesis re-initiation (Mohammad-Qureshi et al., 2008). Whereas loss of eIF2 is incompatible with life, variations in the activity of enzymes that phosphorylate eIF2 or disrupt eIF2B activity are thought to result in neurodegenerative, myocardial, skeletal, and likely other diseases (Fogli and Boespflug-Tanguy, 2006; Balachandran and Barber, 2007; Chen, 2007; Tisdale, 2007; Costa-Mattioli et al., 2009; Jin et al., 2009; Morel et al., 2009; Pavitt and Proud, 2009; Boot-Handford and Briggs, 2010; Saito et al., 2011).

A common procedure to monitor the early eIF2 dependent step in protein synthesis *in vitro* is collection of the eIF2/GTP/met-tRNAi ternary complex where the tRNAi is charged with a labeled or tagged methionine. A labeled met-tRNAi substrate is readily prepared from the eukaryotic tRNA pool by incubation with prokaryotic aminoacyl tRNA synthetase preparations that predominantly or exclusively charge initiator tRNAi relative to internal tRNAmet, followed by RNA re-extraction and precipitation. Inasmuch as the mixed tRNA preparations used for this purpose are total low molecular mass RNA (sRNA) pools, other sRNAs will also co-isolate with labeled met-tRNAi (Henshaw et al., 1980; Centrella and Lucas-Lenard, 1982).

In addition to their initially understood roles in amino acid transfer during protein synthesis and as integral components of 60S ribosomal subunits, sRNAs are now known to control many molecular events. Early studies revealed an important regulatory effect during myoblast differentiation by so-called translational control RNA (tcRNA) on selective heavy chain myosin expression, which was thought to occur in part through effects on eukaryotic protein synthesis initiation factor 3 (Gette and Heywood, 1979; McCarthy et al., 1983; Zezza and Heywood, 1986). In the last decade there has been far more interest in sRNAs, with better definitions of their roles as activators or repressors of gene expression. In this regard, groups of heavily processed sRNAs derived from previously unsuspected regulatory regions of DNA, intervening sequences of mRNA precursors, or tRNAs themselves, are involved in gene silencing, gene product processing, and direct interactions with a variety of regulatory proteins (Okamura and Lai, 2008; Perron and Provost, 2008; Carthew and Sontheimer, 2009; Ghildiyal and Zamore, 2009; Steitz and Vasudevan, 2009; Pederson, 2010). We here report evidence for a previously unappreciated role for a component in the sRNA pool, by which it reduces eIF2 dependent ternary complex formation. As such, it limits a very early step in the assembly of the protein synthesis apparatus and suppresses protein synthesis re-initiation.

2. Materials and methods

2.1 Reagents

L-[³⁵S]methionine, P11 phosphocellulose, DE-52 DEAE cellulose, benzoylated DEAE cellulose (BD-cellulose), G50-Sephadex, mixed cellulose ester HAWP type filters used in

the ternary complex retention assay, and common laboratory reagents were obtained commercially. Proteinase K (*E. album*) was from Merck/EMD and micrococcal nuclease (*S. aureus*) was from Boehringer/Mannheim.

2.2 Cells and tissues

Murine breast carcinoma derived Ehrlich ascites (EAT) cells were used to produce ribosomal salt washes for initiation factor preparation (Henshaw et al., 1980; Wong et al., 1982). Murine EAT cells or total rat liver RNA extracts were used to prepare soluble RNA pools. Murine EAT cells were cultured in spinner flasks at 37° C with Eagle's Minimal Essential Medium supplemented with 20 mM MOPS buffer and 10% newborn calf serum, and maintained at or just below log phase growth rates.

2.3 Initiation factor preparation and cytoplasmic regulators

Murine EAT cells were harvested and lysed in hypotonic solution containing 10 mM KCl, 1.5 mM Mg acetate, 0.5 mM dithiothreitol, 10 mM Tris buffer (pH 7.4) with a Dounce homogenizer and tight fitting pestle. A post-mitochondrial supernatant was prepared by centrifugation at $10,000 \times g$ for 10 minutes at 4° C, and ribosomes were isolated by centrifugation at $160,000 \times g$ for 2 h at 4 C. The pellet was dissolved in a solution containing 500 mM KCl, 0.1 mM EDTA, 5 mM Mg acetate, 1 mM dithiothreitol, 250 mM sucrose, and 5 mM MOPS buffer (pH 7.4) and re-centrifuged at $160,000 \times g$ to obtain a ribosomal salt wash. Salt wash derived proteins were fractionated by ammonium sulfate saturation between 30 and 70%. Active eIF2, monitored by ternary complex formation, was filtered on Sephacryl S-200 in a solution containing 350 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 20 mM MOPS buffer (pH 7.4). Peak fractions of activity were then further purified by 0.35 M to 0.8 M KCl gradient elution in a solution containing 0.1 mM EDTA, 5 mM Mg acetate, 0.1 mM dithiothreitol, 10% glycerol, and 20 mM MOPS buffer (pH 7.4) from P11 phosphocellulose, where it eluted at ~0.6 M. Active material derived from P11 phosphocellulose chromatography was dialyzed and re-fractionated by 0.09 M to 0.35 M KCl gradient elution in the same buffer from DE-52 cellulose, where it eluted at ~0.18 M (xx). To assess cytoplasmic counter regulators of TCI activity, post-mitochondrial supernatants were dialyzed in the same buffer with 0.1 M KCl, and fractionated on P11 phosphocellulose by stepped 0.25 M, 0.43 M, and 1.0 M KCl elution (Henshaw et al., 1980; Wong et al., 1982; Panniers and Henshaw, 1983).

2.4 Labeled met-tRNAi

sRNA preparations as sources of tRNA were incubated with [³⁵S]methionine (1 nmol at >800 Ci/mmol) and 9 U of prokaryotic aminoacyl-tRNA synthetase (*E. coli*, EC ligase subclass 6.1.1) or yeast aminoacyl-tRNA synthetase prepared by standard methods (Muench and Berg, 1966) in a solution containing 10 mM KCl, 10 mM Mg acetate, 4 mM ATP, 100 mM cacodylate buffer (pH 7.0) in a total of 4 ml for 10 minutes at 37° C. The reaction was stopped by dilution in 2.0 M Na acetate at pH 4.35. The samples were extracted twice in phenol containing 50 mM Na acetate and 5 mM Mg acetate, dialyzed against 500 mM NaCl and 50 mM Na acetate (pH 5.0), and then against 20 mM Na acetate (pH 5.0). Recovery studies showed a charging efficiency of ~30% of the total input [³⁵S]methionine (Henshaw et al., 1980; Centrella and Lucas-Lenard, 1982).

2.5 Ternary complex formation assay

Met-tRNAi binding to eIF2 was measured by retention of [³⁵S]methionine-labeled complexes on mixed cellulose ester Millipore filters. Reagents were combined in a solution containing 100 mM KCl, 2 mM Mg acetate, 1 mM dithiothreitol, 0.2 mM GTP, 3.2 mM phosphoenolpyruvate, 8 units of pyruvate kinase and 50 mM MOPS buffer (pH 7.4) in 0.5

ml and incubated for 15 min at 28° C unless otherwise indicated. The reaction was stopped by dilution in 4° C buffer and rapidly strained through filters preconditioned with 0.2% (w/vol) bovine serum albumin to limit nonspecific adherence. Filters were washed 5 times with buffer, dried, and analyzed by liquid scintillation counting. Backgrounds from reactions that omitted eIF2 were less than 1% of the total [³⁵S]met-tRNA_i added to the sample (Henshaw et al., 1980; Centrella and Lucas-Lenard, 1982; Wong et al., 1982).

2.6 sRNA preparations

Total rat liver RNA was prepared from a post-mitochondrial supernatant of homogenized tissue that was then extracted in SDS and phenol and collected by repeated ethanol precipitation. The re-dissolved materials were centrifuged through a 20% to 40% sucrose (weight to volume) gradient for 17 hours at 64,000 × g to separate RNA of various molecular mass classes. Fractions containing the peak of sRNA (4S to 6S RNA) were ethanol precipitated and dissolved in 20 mM sodium acetate (pH 5.0). Material obtained from murine EAT cells was incubated with 500 mM Tris buffer (pH 8.4) for 30 minutes at 37 C to release amino acids from tRNA, re-collected with ethanol, and dissolved in 20 mM Na acetate (pH 5.0). Calf liver and rabbit liver sRNA preparations were obtained commercially. Samples were treated with acid, base, or enzymes for preliminary qualitative analyses, as described in the text.

2.7 BD-cellulose chromatography

sRNAs were diluted 5- to 10-fold in a solution containing 400 mM KCl, 10 mM Mg acetate, and 10 mM Na acetate, pH 4.5, and fractionated on pre-equilibrated BD-cellulose. Preliminary studies revealed that essentially all of the UV light (254 nM) absorbing material and of the [³⁵S]met-tRNA_i within the total sRNA pool adhered to the resin bed, consistent with high RNA binding. Materials adherent to the resin were eluted with a gradient of 400 to 1000 mM KCl prepared in the same buffer. Chromatography derived fractions were sampled to measure optical density at 254 nM, salt, or [³⁵S], concentrated by ethanol precipitation, and re-dissolved in 20 mM Na acetate (pH 5.0) and/or dialyzed against 20 mM Na acetate (pH 5.0) before testing for effects on ternary complex formation (Henshaw et al., 1980; Centrella and Lucas-Lenard, 1982).

2.10 Protein synthesis

Reticulocytes were obtained by cardiac puncture from rabbits induced for 4 days with 25 mg/day of acetylphenylhydrazine. Cells were lysed in hypotonic buffer and lysates were diluted 2 fold in a solution to achieve a final concentration of 80 mM K acetate, 1 mM Mg acetate, 0.2 mM GTP, 1 mM ATP, 10 mM creatine phosphate, 1 mg/ml of creatine phosphokinase, 0.1 mM spermidine, 4.5 mM glucose, 20 mM Tris buffer (pH 7.2), a mixture of 19 amino acids (omitting cysteine (Clemens et al., 1974)) each at 0.1 mM, [¹⁴C]leucine (5 mCi/ml; 300 mCi/mmol) at 1.67 μM, and varying amounts of vehicle or TCI. The mixtures were incubated at 28° C for the times indicated in the figures. Aliquots were spotted onto Whatman 3MM filters, extracted for 5 minutes in hot trichloroacetic acid, washed in ethanol, dried, and analyzed by liquid scintillation counting (Centrella and Lucas-Lenard, 1982).

2.11 Polyribosome profiles

Protein synthesis reactions prepared as indicated above but omitting [¹⁴C]leucine were layered over 20% to 40% (weight to volume) sucrose gradients and centrifuged for 3.5 hours at 274,000 × g. The gradients were fractionated mechanically and optical density was continuously monitored at 254 nM. The 40S to 80S (subunits and monoribosomes) and the multimeric ribosome distribution patterns obtained from control and TCI supplemented

extracts were compared by measuring the relative areas of these regions in the optical density profiles (Pain et al., 1980).

3. Results

3.1 The sRNA pool used for tRNA charging contains an inhibitor of eIF2-dependent ternary complex formation

Early studies with ribosome-derived salt washes used to identify eIF2 activity showed that its ability to form a ternary complex with GTP and [³⁵S]met-tRNA_i, prepared from total sRNA charged with [³⁵S]methionine and prokaryotic (*E. coli*) aminoacyl tRNA synthetase, was directly related to the amounts of either the salt wash derived protein or the [³⁵S]met-tRNA_i preparation at non-saturating levels (Pain et al., 1980). However, when using eIF2 isolated after multiple purification steps, we noticed that ternary complex formation was disproportional to the amount of eIF2 added to the assay. That is, higher amounts of purified eIF2 caused far more ternary complex formation than predicted by the increment added to the reaction in combination with [³⁵S]met-tRNA_i (left panel, Fig. 1A). Ternary complex formation was also disproportional to the amount of [³⁵S]met-tRNA_i where, by contrast to eIF2, high levels were surprisingly inhibitory (right panel, Fig. 1A). This suggested that unfractionated [³⁵S]met-tRNA_i preparations contained a ternary complex inhibitor (TCI) more evident in reactions with limiting amounts of purified eIF2. To test this possibility, unfractionated [³⁵S]met-tRNA_i was chromatographed on BD-cellulose by salt gradient elution. [³⁵S]met-tRNA_i resolved slightly ahead of the bulk of UV light (254 nm) absorbing material and typically increased in specific activity by four-fold or more after chromatography. By contrast, fractions that eluted on the trailing edge of UV absorbance, well resolved from [³⁵S]met-tRNA_i, directly inhibited ternary complex formation by BD-cellulose purified [³⁵S]met-tRNA_i (upper panel, Fig. 1B). Analogous results occurred when the sRNA pool was chromatographed without prior amino acid charging (lower panel, Fig. 1B). Similarly, when [³⁵S]met-tRNA_i was treated to release [³⁵S]methionine before chromatography, TCI again resolved on the trailing edge of UV absorbance (data not shown). By size exclusion chromatography, [³⁵S]met-tRNA_i, TCI activity, and UV absorbance coincided on Sephadex G-50, whereas hemoglobin (*M_r* 17,000) and NaCl resolved in the retained volume (Fig. 1C). When unfractionated and BD-cellulose purified [³⁵S]met-tRNA_i were compared directly for activity, purified [³⁵S]met-tRNA_i was linearly incorporated into ternary complexes (Fig. 1D). Furthermore, ternary complex formation was high and saturable with low concentrations of purified eIF2 and BD-cellulose purified [³⁵S]met-tRNA_i (Fig. 1E).

3.2 TCI affects the rate of ternary complex formation, is independent of GTP regeneration, and reveals the suppressive effect of magnesium

Ternary complex formation with purified components was essentially complete after 5 minutes of incubation. However, the amount of time required to achieve full complex formation increased by 10-fold or more with addition of TCI, and related directly to TCI concentration (Fig. 2A). When TCI was pre-incubated with eIF2, [³⁵S]met-tRNA_i binding was readily suppressed during the first several minutes of ternary complex formation *in vitro*. By contrast, pre-incubation with purified [³⁵S]met-tRNA_i and eIF2 essentially eliminated TCI activity (Fig. 2B), indicating that purified eIF2, at least in its native active state, associates preferentially with met-tRNA_i relative to TCI. Together, these findings reveal that variations in either the amounts of individual components or in incubation conditions might mask or reveal the presence of TCI. In this context, initial studies suggested that the inhibitory effects of magnesium on ternary complex formation, by way of its nucleotide binding potential, was related to high met-tRNA_i concentrations in the assay (Roy et al., 1987). We found that magnesium at 0.5 mM to 2.0 mM enhanced binding by

BD-cellulose purified [³⁵S]met-tRNAi to eIF2. Furthermore, the low level of ternary complex formation that occurred with unfractionated [³⁵S]met-tRNAi, where TCI is replete, remained at all magnesium levels up to 2 mM (Fig. 2C). With regard to nucleotide dependent effects, we then considered if small but sufficient amounts of GDP, which forms a highly stable and non-recycling complex with eIF2, (Siekierka et al., 1982), either in commercial GTP preparations or from GTP hydrolysis, could preferentially sensitize eIF2 to the TCI that accompanies excess met-tRNAi. Purified met-tRNAi binding to eIF2 occurred without GTP, and adding GTP increased binding by 2-fold. Still, as much as a 10-fold increase in GTP did not overcome TCI activity (left panel, Figure 2D). However, the presence of a GTP regenerating system composed of both pyruvate kinase and phosphoenol pyruvate further enhanced ternary complex formation, but again, this was only evident in the absence of TCI (right panel, Fig. 2D).

3.3 TCI occurs in various sources of sRNA

Our initial studies that revealed the presence of TCI in either methionine-charged or deacylated tRNA preparations were performed with commercial rabbit liver tRNA. All sRNA preparations used to date as possible tRNA sources contain a similarly directed TCI. This includes other commercial sources of tRNA from rabbit or calf liver and sRNA isolated from rat liver or from murine EAT cells isolated by repeated phenol extraction and ethanol precipitation (Fig. 3A), albeit with some differences in relative activity. Notably, isolation of TCI by BD-cellulose chromatography increased its relative activity (that is, its inhibitory potential) by at least ten-fold, predicting that inhibition of ternary complex formation did not derive from bulk sRNA alone. At least some portion of TCI activity might result from endogenous charged met-tRNAi within the sRNA pool, which could dilute the specific activity of [³⁵S]met-tRNAi and merely appear to suppress ternary complex formation. To check this, sRNA from growing murine EAT cell lysates, which contains the total complement of charged and uncharged tRNAs, was isolated on sucrose density gradients (left panel, Fig 3B), phenol extracted, and tested for TCI activity before and after amino acid discharging in mild base (0.5 M Tris buffer at pH 8.5), a condition that effectively releases [³⁵S]methionine from [³⁵S]met-tRNAi (Fig. 3C). There were no differences in TCI activity if the sample included the low molecular mass RNA within the 4-6S RNA pool, or the total soluble material from the beginning fractions of the sucrose gradients up to and including the 4-6S RNA pool. Moreover, rather than eliminating its inhibitory effect, treatment with mild base enhanced TCI activity in the 4-6S RNA pool by at least 2 fold (right panel, Fig. 3B). Finally, when rabbit liver sRNA was charged with yeast rather than bacterial aminoacyl tRNA synthetase, TCI activity remained and similarly eluted from BD cellulose (Fig. 3D). Therefore, TCI occurs in sRNA preparations that are charged with methionine by prokaryotic or eukaryotic synthetase, in native sRNA from actively growing eukaryotic cells, and in preparations where prior tRNA charging has been eliminated.

3.4 TCI is sensitive to stringent base hydrolysis but not proteolysis

TCI that occurs in the sRNA preparations is unlikely to result from a small polypeptide that escaped phenol extractions since its inhibitory activity also remained after digestion with proteinase K (Fig. 4A). By contrast, exposure to extremely basic conditions (0.3 M KOH for 2 hours) significantly reduced TCI activity (Fig 4B), consistent with RNA rather than DNA (Li and Breaker, 1999). Even so, TCI dependent inhibition does not appear to depend on major regions of single stranded RNA since its was resistant to short term treatment with micrococcal nuclease (Fig. 4C) under conditions that eliminate translatable mRNA in cell lysates by greater than 95%, but preserves endogenous, translationally competent tRNA (Centrella and Lucas-Lenard, 1982). These findings are consistent with the chemical nature of TCI as a small RNA rather than protein or DNA, which is relatively base resistant (Li and

Breaker, 1999), and suggest that TCI activity may in part depend, functionally or catalytically, on its secondary structure.

3.5 TCI suppresses protein synthesis in vitro

BD-cellulose fractionated TCI potently suppressed amino acid incorporation in cell free lysates prepared from rabbit reticulocytes. Consistent with its time-dependent inhibitory effect on ternary complex formation (Fig. 2A), TCI reduced the rate rather than the extent of amino acid incorporation into hot acid precipitates (Fig. 5A). At earlier times when TCI was active, its inhibitory effect was concentration dependent (Fig. 5B). This paralleled a reduction in heavy polymeric ribosomes (Fig. 5C), such that the ratio of ribosomal subunits and monoribosomes increased relative to multimeric polyribosomes by nearly 10-fold (Fig. 5D).

3.6 The effect of TCI can be reversed by other cellular components

Inasmuch as TCI limits the rate rather than the extent of ternary complex formation and protein synthesis, its effect may be influenced by other intracellular components. To address this, the cytosolic supernatant that remained after high speed centrifugations to collect ribosomes from murine EAT cell lysates was separated into four fractions (A-D in Fig. 6A) by stepped cation exchange chromatography on P11 phosphocellulose. When assayed with BD-purified [³⁵S]met-tRNAi, the bulk of eIF2 found in the ribosome-free supernatant occurred in fraction D in the 0.43 to 1.0 M KCl eluate, whereas fractions A and B, which elute below 0.25 M NaCl, and fraction C, which eluted between 0.25 and 0.43 M NaCl, caused insignificant ternary complex formation. There was an additive effect by the combination of eIF2 and fraction D where ribosome-free eIF2 occurred. When the fractions were assayed with either [³⁵S]met-tRNAi before purification or with BD-cellulose purified [³⁵S]met-tRNAi in the presence of TCI (Fig. 6B), fraction C potently enhanced ternary complex formation by eIF2. Notably, the “anti-inhibitory” effect of fraction C *versus* TCI was dose dependent (Fig. 6C). How this occurs is uncertain, but based on evidence in Fig. 2C, it may not relate directly to eIF2B dependent GDP recycling from eIF2, but rather focus on TCI itself. By contrast, pretreatment of TCI with pyruvate kinase and phosphoenolpyruvate as a phosphate donor before adding it to the principal ternary complex components (eIF2, [³⁵S]met-tRNAi, and GTP), significantly suppressed its inhibitory effect on ternary complex formation (Fig. 6D). Therefore, the effect of TCI on ternary complex formation can be counteracted by a factor that could mask TCI activity in impure eIF2 preparations. Alternately, it could be suppressed by changes in phosphorylation status, perhaps by a cytoplasmic kinase, an effect that is mimicked in a nonspecific way by pyruvate kinase.

4. Discussion

Our current studies provide evidence for a functional ternary complex inhibitor, here termed TCI, which limits met-tRNAi binding to eIF2, disrupts the assembly of heavy polymeric ribosomes, and reduces protein synthesis in eukaryotic cell-free systems. TCI in cell extracts partitions with 4S to 6S sRNA by gradient centrifugation, co-isolates with sRNA by organic extraction, ethanol precipitation, and size exclusion chromatography, and resolves from the bulk of sRNA and from met-tRNAi by ion exchange chromatography. Unlike DNA, TCI is highly sensitive to base hydrolysis. Finally, like tRNA, TCI or its active domain is not susceptible to limited exposure to micrococcal nuclease which rapidly degrades mRNA and DNA, predicting that TCI contains significant secondary structure. Based on its ability to limit met-tRNAi binding to eIF2, it may share significant structural homology with tRNA or other regulatory RNAs such as the original descriptions of tcRNA, a molecule that prevents the translation of myosin heavy chain mRNA, and possibly other mRNAs, prior to myoblast

fusion (McCarthy et al., 1983; Zezza and Heywood, 1986). It clearly differs in function from the 4S small translational control RNA, which is perhaps the same entity as tRNA, in embryonic chick muscle that does not suppress met-tRNAⁱ binding, but rather the downstream step of mRNA binding to the small ribosome subunit (Winkler et al., 1983). Other studies suggest that another small tRNA, which partitions with polyA (+) RNA, helps to delay skeletal muscle cell differentiation, a fate that is reprised by signals generated by demineralized bone matrix (Nathanson et al., 1986; Vanderburg and Nathanson, 1988), but its relationship to TCI is unknown.

Pre-incubation studies predict that TCI competes with met-tRNAⁱ to form a reversible complex with eIF2, albeit with lower affinity than met-tRNAⁱ, since TCI suppresses the rate rather than the ultimate extent of ternary complex formation. The rate limiting effect of TCI also occurs on cell free protein synthesis, where it causes a rapid increase in monomeric ribosomes. It therefore permits polypeptide chain elongation that occurs after a single or minimal round(s) of initiation, and at sufficiently high concentration, effectively blocks re-initiation. These findings predict that changes in the level of TCI relative to met-tRNAⁱ would alter the balance between these molecules and enhance or limit its inhibitory effect. This could occur under various stress-related conditions well associated with the inhibition of protein synthesis, through changes in TCI synthesis, metabolism, or chemical modification. In this regard, we found that the effectiveness of TCI can be controlled in both positive and negative ways. First, the inhibitory effect of TCI on eIF2 activity is enhanced when it is subjected to the mild basic conditions that readily release previously charged amino acids from tRNAs. Even so, an increase in overall uncharged tRNA, and therefore more generalized effects by uncharged tRNAs themselves as a result of amino acid depletion (Kimball and Jefferson, 2005), is unlikely to account directly for TCI activity since TCI partitions in a highly discrete region of the total sRNA profile during chromatography. Moreover, the well known decrease in eIF2 activity which occurs in response to nutritional deprivation and its corresponding increase in uncharged tRNA *in vivo* derive from the eukaryotic downstream kinase related to yeast GCN2 that directly phosphorylates the eIF2 α subunit (Hinnebusch, 1993; Sood et al., 2000). Phosphorylated eIF2 in turn irreversibly binds to and inactivates eIF2B, which disallows GDP release and eIF2 recycling after a round of protein synthesis initiation (Wek et al., 2006). Therefore, although high concentrations of TCI effectively limit eIF2 activity, the time dependent reversible effect of even high amounts of TCI on ternary complex formation largely precludes the irreversible effect of kinase dependent inactivation of eIF2 recycling. Secondly, the inhibitory effect of TCI is significantly reduced by exposure to pyruvate kinase. Whereas TCI is unlikely to be a primary or native substrate of pyruvate kinase, this change in activity may reflect the consequence of an endogenous polynucleotide kinase. Still, pyruvate kinase or other phosphotransferases may have so-called promiscuous secondary effects on less well recognized substrates, which have only more recently been appreciated (Bornscheuer and Kazlauskas, 2004; Khersonsky and Tawfik, 2010). We also found a counteracting effect on TCI activity by a component in the ribosome free cytoplasmic compartment of cultured murine breast EAT cells. This could be accounted for by an authentic endogenous TCI kinase, or perhaps an independent binding partner for either eIF2 or for TCI itself, but not by cytoplasmic eIF2 which isolates independently from this component. Therefore, while TCI is a potent inhibitor of eIF2 dependent ternary complex formation and downstream protein synthesis, intrinsic control mechanisms could modulate its initial effectiveness and vary in response to external signals that are known to enhance or suppress the protein synthetic process.

By molecular sieving chromatography, TCI appears similar in size to tRNA as well as to precursors for endogenous micro RNAs that bind mRNA and silence translation, through mRNA degradation or their interference with other components of the translation machinery

(Carthew and Sontheimer, 2009). Our evidence predicts a distinct effect by TCI, in that it appears to occur through protein (eIF2) rather than RNA binding, although we have not yet formally discounted the possibility that TCI may complex with met-tRNAⁱ and limit its access to eIF2. Moreover, it also remains possible that TCI is itself a micro RNA precursor with a previously unrecognized function upstream of the effects of miRNAs and siRNAs. Again, whereas mild basic conditions enhance its inhibitory potential, the functional domain or component of the TCI found in the 4S to 6S RNA pool may survive and/or be further regulated by the controlled enzymatic processing and regulatory components that produce miRNAs and siRNAs, or some tRNA derived sequences (Pederson, 2010).

In summary, our current studies reveal a novel regulatory effect by a component that we term TCI in the sRNA pool of eukaryotic cells, which suppresses protein synthesis at the level of eIF2 dependent ternary complex formation prior to ribosome engagement. The effect of TCI is reversible and appears sensitive to positive and negative regulation. More studies will be needed to determine if TCI coordinates with or enforces the better understood control mechanisms that occur in response to inflammation, viral, nutritional, or oxidative stresses that converge on eIF2 activity through a small set of kinases that limit its ability to release GDP and re-initiate translation.

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Abbreviations

eIF2	eukaryotic initiation factor 2
sRNA	low molecular mass RNA
met-tRNAⁱ	methionine charged initiator methionyl-tRNA
TCI	ternary complex inhibitor
BD-cellulose	benzoylated DEAE cellulose

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Fig. 1. An inhibitor of eIF2 dependent ternary complex formation occurs in the sRNA pool of eukaryotic cells

A: Ternary complex formation by eIF2, GTP, and met-tRNAi was examined with increasing amounts of eIF2 (left panel) or unfractionated (unfxd) sRNA from rabbit liver charged with [35 S]methionine and *E. coli* aminoacyl tRNA synthetase (right panel). B: sRNA containing [35 S]met-tRNAi (upper panel) or uncharged sRNA (lower panel) were chromatographed on BD-cellulose by 0.1 to 0.4 M NaCl linear gradient elution (dashed line) and the fractions were monitored for optical density at 254 nm (solid line); radioactivity when present (gray circles); and ternary complex formation with BD-cellulose purified (BD-c fxd) [35 S]met-tRNAi (black circles). C: sRNA containing [35 S]met-tRNAi was chromatographed on G50-

Sephadex (superfine) in parallel with hemoglobin (inverse black triangle) and NaCl (dashed line), and the fractions were monitored for optical density at 254 nM (solid line); radioactivity (gray circles); and ternary complex formation with BD-c fxd [³⁵S]met-tRNAi (black circles). D: Ternary complex formation with eIF2 and GTP was compared with unfractionated sRNA containing [³⁵S]met-tRNAi (black circles) or BD-c fxd [³⁵S]met-tRNAi (black squares). E: Ternary complex formation with GTP and increasing amounts of eIF2 was compared with BD-c fxd [³⁵S]met-tRNAi at the levels indicated.

Fig. 2. sRNA derived TCI suppresses eIF2 activity

A: Ternary complex formation with eIF2, GTP and BD-c fxd [³⁵S]met-tRNAi was measured after increasing intervals of incubation with no addition (black circles) or with the amounts of BD-cellulose chromatography derived TCI indicated (black triangles and squares). B: Ternary complex formation with eIF2, GTP and BD-c fxd [³⁵S]met-tRNAi was measured when eIF2 was pre-incubated for 15 minutes with TCI before the addition of GTP and [³⁵S]met-tRNAi (black circles) or when eIF2 was pre-incubated for 15 minutes with [³⁵S]met-tRNAi before the addition of GTP and TCI (black squares) and a secondary incubation for the intervals shown in the left panel with a fixed amount of TCI; or with the amounts of TCI indicated and a 1 minute secondary incubation. C: Ternary complex formation with eIF2 and GTP was compared with unfractionated sRNA containing [³⁵S]met-tRNAi (black circles) or BD-c fxd [³⁵S]met-tRNAi (black squares) and increasing amounts of magnesium, as indicated. D: Ternary complex formation with eIF2 and BD-c fxd [³⁵S]met-tRNAi was compared with increasing amounts of GTP, as indicated in the left panel, without (black circles) or with TCI (black squares); or as indicated in the right panel, without or with pyruvate kinase (PK) or phosphoenol pyruvate (PEP) to regenerate GTP, and TCI.

Fig. 3. TCI occurs with varying sources of sRNA and tRNA synthetase

Ternary complex formation with eIF2, GTP, and BD-c fxd [^{35}S]met-tRNAi was compared with unfractionated sRNA from calf liver (gray circles), rat liver (gray squares), mouse breast cell cultures (gray triangles), rabbit liver (inverse gray triangles); BD-c frxd TCI from three different rabbit liver preparations (black symbols) at the levels indicated. B: A ribosome profile from murine breast EAT cells was prepared by sucrose density gradient centrifugation (left panel); RNA was extracted from the total soluble area below 40S ribosome subunits, or from the 4-6S RNA peak only, and tested for effects on ternary complex formation with eIF2, GTP, and BD-c fxd [^{35}S]met-tRNAi (right panel). C: [^{35}S]met-tRNAi was incubated for 30 minutes at 37°C at pH 8.5, precipitated with ethanol, re-dissolved, and analyzed for optical density at 254 nm and radioactivity relative to the levels before incubation. D: Rabbit liver sRNA charged with [^{35}S]methionine and yeast

aminoacyl tRNA synthetase was chromatographed on BD-cellulose by 0.1 to 0.4 M NaCl linear gradient elution (dashed line) and the fractions were monitored for optical density at 254 nm (solid line) and radioactivity (gray circles) and various fractions were divided into the six groups shown (left panel); ternary complex formation was assayed with the BD-cellulose derived fractions from groups 1-5 (black bars) or with group 2 in combination with increasing amounts of group 6 (black circles), which was essentially devoid of [^{35}S]met-tRNAi (right panel).

Fig. 4. The differential enzymatic and chemical sensitivity of TCI

A: Ternary complex formation with eIF2, GTP, and BD-c frd [35 S]met-tRNAi was compared with increasing amounts of BD-c frd TCI that was pre-treated for 30 minutes at 37°C with water or (black circles) 0.5 mg/ml proteinase K. B: Ternary complex formation with eIF2, GTP, and BD-c frd [35 S]met-tRNAi was compared with vehicle (black circles), BD-c frd TCI that was dialyzed versus 0.02 M sodium acetate (black squares), or pretreated with 0.3 M KOH at 37°C and then dialyzed against 0.02 M sodium acetate before assay (black triangles). C: Ternary complex formation with eIF2, GTP, and BD-c frd [35 S]met-tRNAi was compared with BD-c frd TCI that was pre-treated for 5 minutes at 28°C with calcium dependent micrococcal nuclease, 10 µg/ml 0 minutes at 37°C, or with calcium controls (left panel). The relative potency of micrococcal nuclease was verified by digestion of mRNA, which was virtually eliminated by subsequent analysis in the reticulocyte lysate *in vitro* protein synthesis assay supplemented with [35 S]methionine (right panel).

Fig. 5. TCI suppresses protein synthesis and polyribosome assembly in rabbit reticulocyte lysate
 A and B: Protein synthesis was measured *in vitro* in rabbit reticulocyte lysates supplemented with [^{14}C]leucine and vehicle (black circles) or BD-c frxd TCI (black squares) for the times indicated (panel A); or for 0 (black circles), 30 minutes (black squares), or 90 minutes (black triangles) with the amounts of BD-c frxd TCI indicated (panel B). C: Polyribosome profiles generated by sucrose density gradient centrifugation of rabbit reticulocyte lysates that were supplemented with unlabeled amino acids and either vehicle (0 TCI) or the amounts of TCI indicated, and incubated for 20 minutes at 28° C. D: The relative area of the 40S, 60S and 80S peaks, relative to the area corresponding to the polyribosomes was determined by densitometry in control and TCI supplemented lysates.

Fig 6. Counter-regulation of TCI activity

A: Cell lysates cleared of nuclei and particulate membrane and ribosome components was dialyzed and subjected to stepped cation exchange chromatography on P11 phosphocellulose to produce four fractions, A-D. B: Ternary complex formation with GTP, and BD-c fxd [³⁵S]met-tRNAi was compared in fractions A-D with no addition (white bars); with BD-c fxd TCI (light gray bars); with eIF2 (dark gray bars); or with TCI and eIF2 (black bars). C: Ternary complex formation with eIF2, GTP, and BD-c fxd [³⁵S]met-tRNAi was compared with increasing amounts of fraction C in the absence (black circles) or presence of TCI (black squares). D: Ternary complex formation with eIF2, GTP, and BD-c fxd [³⁵S]met-tRNAi for the times indicated was compared with no additions (black circles); with TCI alone pre-incubated for 30 minutes at 28° C (black triangles); with pyruvate kinase (PK) and phosphoenol pyruvate (PEP) pre-incubated for 30 minutes at 28° (black squares); or with TCI pre-incubated with PK and PEP for 30 minutes at 28° (black inverse triangles).