A role for the eFl4E-binding protein 4E-T in P-body formation and mRNA decay

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4E-transporter (4E-T) is one of several proteins that bind the mRNA 5’cap-binding protein, eukaryotic initiation factor 4E (eIF4E), through a conserved binding motif. We previously showed that 4E-T is a nucleocytoplasmic shuttling protein, which mediates the import of eIF4E into the nucleus. At steady state, 4E-T is predominantly cytoplasmic and is concentrated in bodies that conspicuously resemble the recently described processing bodies (P-bodies), which are believed to be sites of mRNA decay. In this paper, we demonstrate that 4E-T colocalizes with mRNA decapping factors in bona fide P-bodies. Moreover, 4E-T controls mRNA half-life, because its depletion from cells using short interfering RNA increases mRNA stability. The 4E-T binding partner, eIF4E, also is localized in P-bodies. 4E-T interaction with eIF4E represses translation, which is believed to be a prerequisite for targeting of mRNAs to P-bodies. Collectively, these data suggest that 4E-T interaction with eIF4E is a priming event in inducing messenger ribonucleoprotein rearrangement and transition from translation to decay.

Introduction

The regulation of mRNA biogenesis and decay plays an important role in the control of gene expression. mRNA decay regulates protein levels, eliminates aberrant mRNAs, and is an essential host defense mechanism during viral infection (Coller and Parker, 2004). There are three major mRNA decay pathways (Coller and Parker, 2004; Parker and Song, 2004). The general pathway involves deadenylation followed by decapping of the mRNA. In yeast, transcripts are primarily degraded in a 5’→3’ direction by the Xrn1 exonuclease (Muhlrad et al., 1995; Schwartz and Parker, 1999; Coller and Parker, 2004; Parker and Song, 2004), whereas in higher eukaryotes, mRNAs mostly are eliminated in a 3’→5’ fashion by the exosome after deadenylation (Chen et al., 2001; Hilleren et al., 2001; Wang and Kiledjian, 2001; Wilusz et al., 2001; Mukherjee et al., 2002; Tourriere et al., 2002). mRNA degradation also can occur through a second pathway that involves endonucleolytic cleavage of mRNAs (Schoenberg and Chernokalskaya, 1997), and in addition, through specialized mechanisms, including nonsense-mediated decay and nonstop decay (for reviews see Wilusz et al., 2001; Tourriere et al., 2002; Coller and Parker, 2004; Parker and Song, 2004).

Mammalian mRNA decay usually occurs in two steps, deadenylation followed by decapping. Most mammalian mRNAs studied are the target of degradation by the 3’-5’ exosome (Chen et al., 2001; Mukherjee et al., 2002; Wang and Kiledjian, 2001). Nonetheless, decapping activity similar in function to the yeast Dcp1p has been detected in HeLa cells (Gao et al., 2001); mammalian homologues of Dcp1/2 (Lykke-Andersen, 2002; Wang et al., 2002), the Lsm proteins (Achsel et al., 1999), and Dhh1p (Smillie and Sommerville, 2002) also exist. Furthermore, Xrn1 homologues have been identified in mammals (Bashkirov et al., 1997; Ingelfinger et al., 2002). However, it has not been demonstrated unequivocally whether deadenylated-decapped mRNAs are subject to 5’ or 3’ exonucleolytic decay in mammalian cells in vivo (discussed in Wang and Kiledjian, 2001; Mukherjee et al., 2002; Wilusz and Wilusz, 2004).
The mRNA 5' cap structure and the 3' polyA tail play important roles in the decapping process (Wilusz et al., 2001; Coller and Parker, 2004). The polyA inhibits decapping, likely through the polyA binding protein (PABP) interaction with eIF4G, a component of the eIF4F cap-binding complex (Coller and Parker, 2004). eIF4F also contains the cap binding protein, eukaryotic initiation factor 4e (eIF4E), and the RNA helicase, eIF4A (Gingras et al., 1999). The interaction of PABP with eIF4G enhances the binding of eIF4F to the mRNA (Kahvejian et al., 2005), and thus, hinders the access of the decapping complex to the cap. Consistently, translation and decapping are antagonistic. Consistent with this idea, mutations in eIF4E and eIF4G can lead to an increase in decapping in yeast (Schwartz and Parker, 1999). Importantly, decapping requires the removal of eIF4F from the cap structure, which, in turn, necessitates a transition from a translationally active messenger RNA (mRNP) to one destined for decay. This transition entails the replacement of initiation factors on the mRNA with decapping factors (Tharun and Parker, 2001; Coller and Parker, 2004; Parker and Song, 2004; Teixeira et al., 2005). The switch from translation to decay occurs concomitantly with aggregation of mRNPs into distinct cytoplasmic foci, termed processing bodies (P-bodies), in yeast (Sheth and Parker, 2003), or GW bodies (Eystathioy et al., 2003) or Dcp1a bodies in mammals (Cougot et al., 2004); these are referred to as P-bodies throughout this article. The decapping enzyme, Dcp1/2, and decapping associated factors, Lsm1-7, Dhh1p/p54, and Xrn1, are concentrated in these foci (Ingelfinger et al., 2002; van Dijk et al., 2002; Sheth and Parker, 2003; Cougot et al., 2004; Liu et al., 2004). However, little is known about the recruitment mechanism of mRNAs to P-bodies, or how initiation factors are exchanged with decapping enzymes.

We reported earlier the cloning of 4E-transporter (4E-T), which interacts with eIF4E through a conserved eIF4E recognition motif (YXXXXL-Φ, where Φ is a hydrophobic amino acid) that is also found in eIF4G and 4E-BP. 4E-T is a nucleocytoplasmic shuttling protein that mediates the nuclear import of eIF4E through the importin α/β pathway. At steady state, 4E-T is predominantly cytoplasmic and is concentrated in bodies that conspicuously resemble the recently described P-bodies (Dostie et al., 2000a; Sheth and Parker, 2003). Here, we report that 4E-T colocalizes with eIF4E and decapping factors in bona fide P-bodies and controls mRNA turnover. Significantly, depletion of 4E-T from cells enhances mRNA stability.

**Results**

**4E-T colocalizes with decapping factors**

To determine whether 4E-T associates with P-bodies, the localization of 4E-T and P-body components was analyzed by immunofluorescence in HeLa cells. Cells transfected with HA–4E-T were analyzed by double-label immunofluorescence microscopy using anti-HA antibody and an affinity-purified anti-Dcp1a antibody that recognizes the endogenous protein (see Materials and methods). Transfected HA–4E-T and endogenous Dcp1a colocalized in the cytoplasm in discrete foci that strongly resemble P-bodies (Fig. 1 A). Similar results were obtained with a GFP-tagged 4E-T (Fig. 1 B). To show that endogenous 4E-T also localizes to P-bodies, its colocalization with Me31B was studied (Me31B is the Drosophila homologue of the human decapping factor Dhh1p/p54, and similar to Dcp1a, is an established marker of P-bodies [Sheth and Parker, 2003; Tseng-Rogenski et al., 2003]; Me31B shares 71% identity with Dhh1p/p54 and was shown to form cytoplasmic granules in germ line cells [Nakamura et al., 2001]). HeLa cells transfected with myc-EYFP-Me31B were stained with an affinity-purified anti–4E-T antibody, and analyzed by microscopy. Endogenous 4E-T colocalizes with myc-EYFP-Me31B in discrete cytoplasmic foci (Fig. 1 C). Thus, endogenous and transfected 4E-T colocalize with bona fide decapping factors in cytoplasmic foci, which fulfill the criteria of P-bodies.

**4E-T down-regulates cap-dependent translation**

In the yeast *Saccharomyces cerevisiae*, targeting of mRNAs to P-bodies is believed to occur as a consequence of cessation of translation (Schwartz and Parker, 1999; Tharun and Parker, 2001; Teixeira et al., 2005). Thus, it is conceivable that changes in mRNP composition that lead to translational arrest result in targeting the mRNA to P-bodies in mammals. 4E-T interacts with eIF4E through a conserved binding site, which also is found in eIF4G (Mader et al., 1995), and thus, might promote targeting of mRNAs to P-bodies by inhibiting translation initia-

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**Figure 1.** 4E-T colocalizes with decapping factors in P-bodies. (A and B) Transfected 4E-T colocalizes with endogenous Dcp1a. HeLa cells transfected with HA–4E-T (A) or GFP–4E-T (B) were stained with anti-Dcp1a to visualize endogenous Dcp1a. The localization of HA–4E-T was determined by indirect immunofluorescence with anti-HA (Covance) and Alexa Fluor 594 anti–mouse IgG. Dcp1a was revealed with anti-Dcp1a and either Alexa Fluor 488 anti–rabbit IgG (A) or Alexa Fluor 594 anti–mouse IgG (B). The colocalization of HA–4E-T or GFP–4E-T with Dcp1a appears yellow (right). (C) Endogenous 4E-T colocalizes with transfected myc-EYFP-Me31B. HeLa cells were transfected with myc-EYFP-Me31B and colocalization with endogenous 4E-T was performed with anti–4E-T and Texas Red–conjugated anti–rabbit IgG. Colocalization appears yellow (right).
To address this possibility, the inhibitory effect of 4E-T on translation was examined in vivo. HeLa cells infected with recombinant vaccinia virus vTF7-3 (to synthesize the T7 RNA polymerase in the cytoplasm) were cotransfected transiently with plasmid DNA containing a 4E-T cDNA and a bicistronic reporter construct expressed from a T7 promoter. The bicistronic reporter construct consists of the luciferase (LUC) and chloramphenicol acetyl transferase (CAT) cistrons, separated by the poliovirus internal ribosome entry site (IRES; Pause et al., 1994) (Fig. 2 A). Translation of the LUC cistron proceeds in a cap-dependent manner, whereas translation of the CAT cistron is cap-independent and serves as a surrogate control for mRNA levels. (Note that quantification of the RNA by Northern blotting could not be performed because the vaccinia virus T7 system produces heterogeneous RNA transcripts, generating smeared bands [Fuerst and Moss, 1989]. This is attributed to the lack of an authentic T7 RNA polymerase termination sequence. However, this has no deleterious effect on mRNA turnover or translation [Fuerst and Moss, 1989].) Only a fraction (5–10%) of T7-transcribed RNAs are capped by the vaccinia-capping enzyme (Fuerst and Moss, 1989). However, uncapped RNA is translated inefficiently in vivo and in vitro (Both et al., 1975; Shatkin, 1976; Sonenberg et al., 1980; Fuerst and Moss, 1989; Peng and Schoenberg, 2005; and unpublished data). Thus, even if only a small fraction of the T7-transcribed RNAs is capped in the T7 vaccinia virus system, the uncapped products largely are untranslated (~0.4–1% of uncapped compared with capped RNA; unpublished data). All constructs were expressed to similar levels (Fig. 2 B, bottom panel, compare lanes 2–4). The binding of 4E-T to elf4E was demonstrated by Far Western blotting with [32P]HMK-elf4E (Fig. 2 B, lane 2, top panel). The 4E-T mutant, Y30A, which is mutated in the YXXXXLΦ motif, did not bind to elf4E, as expected (Fig. 2 B, lane 3, top panel). LUC expression was decreased significantly by overexpression of wild-type 4E-T (Fig. 2 C, left, top panel), whereas CAT activity remained relatively unchanged (Fig. 2 C, left, bottom panel). In contrast, the Y30A mutant of 4E-T did not affect LUC or CAT expression. Therefore, overexpression of wild-type 4E-T, but not the mutant Y30A, strongly inhibited (88%) translation (Fig. 2 C). In conclusion, overexpression of 4E-T in HeLa cells results in the repression of translation only when bound to elf4E.

eIF4E localizes to P-bodies

Because 4E-T localizes to P-bodies, and because it interacts with elf4E, it was pertinent to ask whether elf4E also localizes to P-bodies, and whether this localization requires 4E-T. To this end, we performed immunofluorescence analysis of endogenous elf4E in HeLa cells using a monoclonal antibody, which recognizes elf4E in the cytoplasm (Kimball et al., 2003). Although elf4E is distributed throughout the cytoplasm, it is...
Figure 3. **eIF4E colocalizes with 4E-T, Dcp1a, and Dcp2 in P-bodies.** (A) HeLa cells (CCL2) were fixed and the localization of eIF4E was determined by indirect immunofluorescence with mouse anti-eIF4E monoclonal antibody and Alexa Fluor 594 anti–mouse IgG. The localization of 4E-T was examined with anti–4E-T and that of Dcp2 with anti-Dcp2 and Alexa Fluor 488 anti–rabbit IgG. The colocalization of these factors appears yellow in the merged image. (B) The colocalization of endogenous eIF4E with 4E-T and Dcp1a was examined with anti–4E-T and anti-Dcp1a in HeLa S3 as above. The left panel

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concentrated in foci reminiscent of P-bodies (Fig. 3 A). This staining pattern is observed in two different HeLa cell sublines: HeLa CCL2 and HeLa S3 (Carter et al., 1995), but with qualitative differences (Fig. 3, A and B–E, respectively), and in mouse embryo fibroblasts (not depicted). Furthermore, transiently transfected HA-eIF4E also displays a similar cytoplasmic distribution pattern (see below, Fig. 4 A).

Double-label immunofluorescence analysis shows that eIF4E co-localized with 4E-T and Dcp2 in P-bodies (Fig. 3 A). eIF4E exhibited a more pronounced association with P-bodies in CCL2 (Fig. 3 A) as compared with HeLa S3 (Fig. 3 B). eIF4E co-localized with Dcp1a and 4E-T in P-bodies, albeit staining of eIF4E in these structures is not as apparent as the staining of 4E-T and Dcp1a in HeLa S3 (Fig. 3 B). Strikingly, overexpression of GFP–4E-T resulted in enhanced staining of eIF4E in P-bodies (Fig. 3 C). Because localization of eIF4E in P-bodies was more prominent, and the foci appeared larger and brighter in GFP–4E-T overexpressing cells than in parental cells, these data suggest that 4E-T might tether eIF4E to P-body structures. This effect is illustrated in a graph plotting the intensity of the eIF4E signal (red channel) in two P-bodies marked by an arrow in cells overexpressing 4E-T levels (Fig. 3 D, right panel) and in cells expressing endogenous 4E-T levels (Fig. 3 D, left panel; the respective P-bodies traversed by the arrow are circled in the adjacent panel for comparison). The signal intensity of eIF4E was approximately twofold higher in cells overexpressing 4E-T as compared with endogenous 4E-T levels, when P-bodies of weaker intensity were selected from cells overexpressing 4E-T to avoid signal saturation of the plot. Similar results were obtained with a monoclonal antibody against eIF4E from Transduction Laboratories (610270), and with a monoclonal antibody (10C6), which recognizes eIF4E predominantly in the nucleus (Lejzkowicz et al., 1992; Dostie et al., 2000b; unpublished data). The enhanced eIF4E signal is not due to leakage of the FITC signal, because no significant signal was detected in the red channel when cells transfected with GFP–4E-T were stained with the secondary antibody alone (Fig. 3 E).

To determine whether the association of eIF4E with P-bodies requires its interaction with 4E-T, we performed immunofluorescence analysis using wild-type HA-eIF4E and a mutant lacking the 4E-T binding site (eIF4E W73A) (Dostie et al., 2000a). Low eIF4E cDNA amounts were transfected transiently into HeLa cells to obtain a predominantly cytoplasmic distribution pattern (Dostie et al., 2000a) and P-body localization (Fig. 4 A). Cytoplasmic eIF4E levels in HA-eIF4E–expressing cells were similar to endogenous levels. However, a more pronounced cytoplasmic foci staining was observed in ~40% of the transfected cells, presumably because more eIF4E is expressed in these cells. In contrast, HA-eIF4E W73A did not localize to P-bodies, and was distributed uniformly between the cytoplasm and the nucleus (Fig. 4 A). Therefore, loss of 4E-T interaction (and also interaction with eIF4G and 4E-BPs) (Mader et al., 1995) leads to deregulated cellular localization, which results in diffuse staining throughout the cell. In addition, because the HA–4E-T (Y30A) mutant, which is defective in eIF4E binding (Dostie et al., 2000a), still localizes to P-bodies, these results suggest that 4E-T is required for the association of eIF4E with P-bodies, but not vice versa (see also Fig. 5 C).

Figure 4. eIF4E requires interaction with 4E-T for localization to P-bodies, whereas the other components of the eIF4F complex are excluded from P-bodies. [A] HeLa cells were transfected with HA-eIF4E wt, HA-eIF4E W73A, or HA–4E-T Y30A. Localization of proteins was studied by indirect immunofluorescence with anti-HA (Covance) and Alexa Fluor 594 anti-mouse IgG. [B and C] HeLa cells were transfected with GFP-Dcp1b and staining of endogenous eIF4GI (B) or eIF4A (C) was detected with anti-eIF4GI and Texas Red conjugated anti–rabbit IgG or with anti-eIF4A and Alexa Fluor 594 anti–mouse IgG. (D) HeLa extract (50 and 100 µg) was resolved by SDS-8% PAGE and a Western blot was performed with rabbit anti–eIF4GI polyclonal antibody. For eIF4A detection, proteins were resolved by SDS-10% PAGE and Western blot was performed with a mouse anti-eIF4A monoclonal antibody.

Demonstrates endogenous eIF4E localization in HeLa S3. Zoomed images (right panel) display colocalization of endogenous eIF4E and Dcp1a in P-bodies. Some of the more distinct P-bodies are indicated by arrowheads. [C] HeLa cells were transfected with GFP–4E-T and the staining of endogenous eIF4E was performed with anti-eIF4E antibody as described above. [D] The intensity of fluorescence of endogenous eIF4E in P-bodies from GFP–4E-T–transfected cells [from panel C] was compared against the intensity of fluorescence of eIF4E from nontransfected cells, which is indicated by the arrow. The P-bodies along the path of the arrow are circled in the adjacent panel for comparison for better visibility. The signal was allowed to bleach before quantification to avoid a saturated signal. The graphs plot the intensity of fluorescence against the distance (µm) traversed by the arrow. [E] HeLa cells transfected with GFP–4E-T were incubated with Alexa Fluor 594 anti–mouse IgG (M-8594) without previous incubation with anti-eIF4E antibody to demonstrate the integrity of the green filter.
Because eIF4E is part of the heterotrimeric eIF4F complex, it was important to determine whether eIF4A and eIF4G also localize to P-bodies. Because 4E-T and eIF4G are expected to compete for binding to eIF4E, no other eIF4F complex components—with the exception of eIF4E—were expected to localize to P-bodies. To investigate this, the localization of endogenous eIF4GI and eIF4A was studied in HeLa cells transfected with GFP-Dcp1b. Western blot analysis with anti-eIF4A, which recognizes eIF4AI and eIF4AII (Edery et al., 1983), and anti-eIF4GI revealed protein bands corresponding to the expected size of the proteins (Fig. 4 D). Immunofluorescence analysis with these antibodies shows that eIF4GI and eIF4A do not localize to P-bodies because neither protein colocalizes with the P-body marker, GFP-Dcp1b (Fig. 4, B and C). Similar results were obtained using two other anti-eIF4GI antibodies (unpublished data). Thus, eIF4E, but neither eIF4GI nor eIF4A, localized to P-bodies in vivo. It is highly likely that eIF4GII behaves in a similar manner, because it exhibits very similar properties to eIF4GI (Gradi et al., 1998).

Figure 5. Depletion of 4E-T from HeLa cells results in disappearance of decapping factors from P-bodies. [A] HeLa cells were transfected with siRNA against 4E-T or control siRNAs (4AIII inverted or 4E-T inverted). 48 h after transfection, cells were split into chamber slides for immunofluorescence analysis [see (C)] and into a 6-cm dish for Western blot analysis. 60 h after transfection, protein cell extracts were prepared and 20 μg of extract was resolved by SDS-10% PAGE. Western blotting was performed with rabbit anti–4E-T and anti-eIF4E antibodies, or mouse anti–β-actin monoclonal antibody (Sigma-Aldrich). 4E-T levels were quantified against β-actin, which served as a loading control, and the level of protein in the negative control (4AIII inverted) transfected cells was set as 100. (B) HeLa extract (20 μg) as in (A) was resolved by 10% SDS-PAGE and Western blotting was performed with rabbit anti-Dcp1a and anti-Xenopus p54 (Xp54) antibodies, or monoclonal mouse anti-eIF4E (SK) and anti–β-actin antibodies. (C) HeLa (CC2) cells were transfected with control siRNA (4AIII inverted) or 4E-T siRNA and indirect immunofluorescence was performed 60 h after transfection with rabbit anti–4E-T and anti-Dcp1a antibodies, or monoclonal mouse anti-eIF4E antibody. (D) HeLa S3 cells were transfected with 4E-T siRNA or control siRNA (4AIII inverted). 24 h after transfection, cells were transfected with myc-EYFP-Me31B; 36 h later, extracts were collected for SDS-PAGE analysis or fixed for immunofluorescence. Cell extracts were resolved by 10% SDS-PAGE and Western blot analysis with rabbit anti–4E-T, or monoclonal anti-myc (9E10) and anti–β-actin antibody. Indirect immunofluorescence was performed to assess the localization of 4E-T, and the localization of myc-EYFP-Me31B was assessed by direct immunofluorescence. The right most panels show the enlarged bordered image.
occurred in 4E-T–depleted cells as compared with nonspecific siRNA controls (Fig. 5, A and B).

The effect of 4E-T depletion on Me31B localization also was examined (Fig. 5 D). The tagged version of Drosophila Me31B was used because the antibody against endogenous p54, used in Fig. 5 B for Western analysis, is not suitable for immunofluorescence. HeLa cells transfected with 4E-T or control siRNAs were transfected with the myc-EYFP-Me31B plasmid, and analyzed by Western blotting and double-label immunofluorescence (Fig. 5 D). 4E-T levels were reduced efficiently in 4E-T siRNA-treated cells, and the myc-EYFP-Me31B protein was expressed in cells treated with control or 4E-T siRNAs (left panel). In control cells, myc-EYFP-Me31B was predominantly cytoplasmic and concentrated in P-bodies (right panel). However, when 4E-T levels were reduced, Me31B exhibited a diffuse staining pattern throughout the cytoplasm and reduced or no staining in P-bodies (right panel). Thus, reduction of 4E-T protein levels causes mislocalization of eIF4E and decapping factors away from P-bodies.

**Cycloheximide inhibits localization of 4E-T to P-bodies, whereas leptomycin B treatment does not affect its localization**

In yeast and mammalian cells, cycloheximide treatment releases decapping factors from P-bodies (Sheth and Parker, 2003; Cougot et al., 2004). Cycloheximide inhibits mRNA translation at the elongation step, which prevents mRNA degradation because of its protection by ribosomes (Ross, 1995). To study whether 4E-T localization to P-bodies is dependent on mRNA availability, the effect of cycloheximide on the localization of 4E-T was examined. HeLa cells were treated with cycloheximide (10 μg/ml), and analyzed by immunofluorescence with anti-Dcp1a and anti-4E-T antibodies. A near complete loss of 4E-T and Dcp1a from P-bodies was observed after 30 min of cycloheximide treatment (Fig. 6 A); this suggested that the accumulation of 4E-T in P-bodies is dependent on mRNA availability. This effect is not due to a reduction in 4E-T protein levels. Western blot analysis demonstrates that the levels of 4E-T and Dcp1a remain unchanged throughout the cycloheximide treatment (Fig. 6 B). Similar results were obtained in HeLa cells that were transfected transiently with HA-4E-T (Fig. 6 C, middle panel).

Leptomycin B (LMB) is an inhibitor of chromosome region maintenance-1–dependent protein nuclear export. We previously reported that 4E-T is a shuttling protein that exits the nucleus through the chromosome region maintenance-I pathway (Dostie et al., 2000a). Because Dhh1p/p54 also shuttles between the nucleus and the cytoplasm (Smillie and Sommerville, 2002), it is possible that nuclear export is required for the localization of 4E-T and decapping factors to P-bodies. To test this hypothesis, the effect of LMB on the localization of 4E-T and Dcp1a to P-bodies was studied. LMB treatment resulted in the accumulation of 4E-T in the nucleus, but did not affect its association with P-bodies (Fig. 6 C, bottom panel). The distribution of Dcp1a also was unaffected by the drug (bottom panel). Thus, the presence of 4E-T in P-bodies is dependent on mRNA, but is independent of its nucleocytoplasmic shuttling.

**4E-T knockdown increases mRNA half-life**

The results described above suggest that 4E-T might play a role in promoting mRNA degradation. To test this hypothesis, we analyzed the turnover of several mRNAs in 4E-T–depleted and

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**Figure 6. Effect of cycloheximide and LMB on 4E-T localization in P-bodies.** [A] HeLa cells were treated with cycloheximide (CHX; 10 μg/ml) for 30 min, and immunofluorescence staining of 4E-T and Dcp1a were examined as described above. [B] HeLa cell extract [20 μg] treated with cycloheximide (CHX; 10 μg/ml) for 30 and 60 min was resolved by 10% SDS-PAGE and Western blot analysis with rabbit anti–4E-T and anti-Dcp1a, or monoclonal mouse β-actin was performed. [C] HeLa cells were transfected with HA–4E-T. 36 h after transfection, the medium was replaced with fresh medium (control) or medium containing CHX [100 μg/ml], and cells were incubated for 3 h before fixation. Alternatively, media containing LMB (5 ng/ml) was added to cells and incubated for 5 h before fixation. The colocalization of HA–4E-T was determined by indirect immunofluorescence with anti-HA (Covance) and that of Dcp1a with anti-Dcp1a antibody as described above. The colocalization of HA–4E-T and Dcp1a appears yellow.
control cells. A β-globin reporter that is under the control of a tetracycline-responsive promoter was fused at its 3’ end to one of two distinct adenine/uridine rich elements (AREs) (Stoecklin et al., 2004), GM-CSF or c-fos. The reporter gene—either lacking or containing the ARE—was cotransfected with a plasmid encoding the activator of transcription (pTet-Off) into HeLa cells that were transfected previously with 4E-T or control siRNA. Doxycycline was added to block transcription and RNA was collected at the indicated times and analyzed by Northern blotting. As expected, β-globin lacking an ARE remained stable throughout the doxycycline treatment, whereas β-globin containing the GM-CSF or c-fos AREs were degraded rapidly, as was reported previously (Chen et al., 1995) (Fig. 7, A and B). However, 4E-T diminution in HeLa cells resulted in significant stabilization (>2-fold) of β-globin/GM-CSF and β-globin/c-fos ARE-containing mRNA, with no effect on β-globin reporter mRNA lacking an ARE (Fig. 7, A and B). Western blot analysis demonstrates that 4E-T protein levels were diminished efficiently in HeLa cells that were transfected with the reporter plasmids and in the presence of doxycycline (Fig. 7 C). The effect of 4E-T depletion on endogenous c-fos mRNA turnover also was analyzed. Similar to transfected β-globin containing the destabilizing element of c-fos, endogenous c-fos was stabilized in the absence of 4E-T (unpublished data).

The stability of the labile p21 mRNA, which contains an ARE (Lal et al., 2004), also was examined in 4E-T–depleted cells. Western blotting analysis demonstrates that 4E-T RNAi strongly reduced the amount of 4E-T (>80%, Fig. 7 D). p21 mRNA stability was determined by Northern blot analysis after actinomycin D treatment to inhibit de novo transcription.

Figure 7.  siRNA-mediated depletion of 4E-T affects mRNA turnover. HeLa cells were transfected with siRNA against 4E-T or a control (Ctrl) siRNA (4AIII inverted). Protein and RNA were harvested for use in Western (C and D) and Northern (A, B, and E) analysis, respectively. (A and B) HeLa cells were transfected with siRNA against 4E-T or with control siRNA and later cotransfected with pTet-β-globin or pTet-β-globin c-fos or GM-CSF ARE and pTet Off. Cells were treated with doxycycline [1 µg/ml] ~72 h after transfection to block transcription. Total RNA was isolated at 0.5, 2, 3, and 4 h for β-globin/GM-CSF ARE assays (A) and 0.5, 1.5, 3, and 6 h for β-globin/c-fos ARE assays (B) and analyzed by Northern blot. GAPDH served as a loading control. mRNA half-lives were calculated from Northern blots and normalized against GAPDH levels. (C) Protein extracts were collected at 6 h after treatment with doxycycline [1 µg/ml] from cells that were transfected with the reporter plasmid and pTet Off and resolved by SDS-PAGE. Lanes 1 and 4: Glo β-globin Gmc; lanes 2 and 5: fos = β-globin/c-fos ARE + pTet Off; lanes 3 and 6: GM = β-globin/GM-CSF ARE + pTet Off. (D) Protein extracts were collected at the latest time point of actinomycin D treatment and resolved by 10% SDS-PAGE. Western blot analysis was done with anti–4E-T and anti–β-actin antibodies. NT = nontransfected. (E) At 48 h after transfection, the half-lives of p21 mRNA were assessed by using actinomycin D [5 µg/ml] for the indicated amount of time. Total RNA (5 µg) was resolved on 1.3% formaldehyde gel and analyzed by Northern blottin. 28S levels and 18S levels served as a loading marker. mRNA half-lives were calculated from Northern blots and normalized against 32P-labeled 18S levels and plotted on a graph with the zero time point set at 100.
p21 mRNA levels were quantified in cells that were treated with 4E-T siRNAs and in nondepleted cells, and normalized to 18S rRNA levels (Fig. 7 E). 4E-T depletion resulted in a modest (~1.5 fold), but significant, increase in the mRNA half-life (Fig. 7 E). The difference in p21 mRNA stability achieved with 4E-T depletion is related inversely to that obtained when the mRNA stabilizer, HuR, is depleted from cells (~2-fold decrease; Lal et al., 2004). Thus, 4E-T promotes degradation of mRNAs that contain an ARE in their 3′ untranslated region.

Discussion

Here we report a new role for 4E-T in mammalian mRNA degradation. We demonstrate that 4E-T inhibits translation, colocalizes with decapping factors in cytoplasmic P-bodies, and decreases mRNA stability. 4E-T interacts with eIF4E and colocalizes with decapping factors in cytoplasmic P-bodies, degradation. We demonstrate that 4E-T inhibits translation, and precedes P-body formation. Recent studies have implied that the 5′-3′ exonucleolytic pathway, is present in P-bodies. Recent studies have implied that the 5′-3′ and 3′-5′ pathways converge at these sites. For instance, mRNA decay enzymes involved in 5′-3′ and 3′-5′ decay are recruited by the ARE binding proteins, tristetraprolin and butyrate response factor (Lykke-Anderson and Wagner, 2005). Moreover, tristetraprolin, which mediates ARE degradation by recruiting the exosome (Chen et al., 2001), also is found localized in P-bodies (Kedersha et al., 2005). Because the disappearance of P-bodies with 4E-T RNAi was not synonymous with complete stabilization of p21 mRNA, it is possible that this process occurs outside of P-bodies or that its deadenylation/decay occurs by way of a distinct pathway from c-fos and GM-CSF mRNAs.
In conclusion, the interaction of 4E-T with eIF4E in cells has two consequences: import of eIF4E to the nucleus (Dostie et al., 2000a) and the targeting of eIF4E to sites of mRNA decay. The function of eIF4E in the nucleus is under investigation; a proposed nuclear function of eIF4E is to export a subset of mRNAs from the nucleus (Lai and Borden, 2000; Rousseau et al., 1996). The dual role of 4E-T also might serve to sequester the limiting factor in translation initiation, eIF4E, as a means of maintaining translational homeostasis. It will be important to examine how 4E-T is regulated. It is reported that 4E-T is a phosphoprotein (Pyronnet et al., 2001); therefore, it would be interesting to know what pathways regulate its phosphorylation and activity.

While this paper was under review, two reports were published which demonstrated that eIF4E is found in P-bodies (Andrei et al., 2005; Kedersha et al., 2005), and that 4E-T is also concentrated in P-bodies (Andrei et al., 2005).

**Materials and methods**

**Plasmids and cell culture**

HA-4E-T, HA-4E-T (Y30A), HA-eIF4E, HA-eIF4E (W73A) (Dostie et al., 2000a), and HA-eIF4E (W73A) (Cougot et al., 2004) were described previously. GFP-4E-T was generated by subcloning the coding region of 4E-T from pcDNA3-4E-T (Dostie et al., 2000a) into pTER143 (provided by G. Pavlakis, National Cancer Institute, Frederick, MD). Myc-EYFP-Me31B plasmid was a gift from A. Nakamura (University of Tsukuba, Tsukuba, Japan).

**Construction of tetracycline-regulated β-globin expression plasmids**

The starting plasmid, CMV-glo-SPA, which contains the human β-globin gene with the 5′ untranslated region and polyadenylation site replaced with a multiple cloning site and a strong synthetic polyadenylation element (SPA), was described previously (Das Gupta et al., 1998). The first generation tetracycline-regulated globin construct pTet-O-βAc-glo-SPA was prepared by digesting CMV-glo-SPA with HindIII, endfilling with Klenow fragment DNA polymerase, then digesting with NcoI. The globin-SPA fragment was gel purified and cloned into pGTEP[O]dAcluc3 (provided by J. Garcia-Sanz, Universidad Autónoma, Madrid, Spain) in which the LUC insert was removed by digesting with BamHI, followed by endfilling with Klenow fragment DNA polymerase plus digestion with NcoI. The c-fos ARE was recovered from the plasmid pB8B-AREc-fos (provided by A.-B. Shyu, University of Texas Medical School, Houston, TX) by PCR amplification with primers BBBARE1 (5′-CGCCTTAGAC-AAGGGGCTTCATGATGTC-3′) and BBBARE2 (5′-GCGTCCTAGCT-CAAGGGGCTTCATGATGTC-3′) and BBAARE3 (5′-CCCTCTAGAGCTGGTTATTGTGCTGTCTCA-3′) and BBAARE4 (5′-CCCTCTAGAGCTGGTTATTGTGCTGTCTCA-3′). The PCR products were digested with XbaI and inserted into XbaI-digested Tet-O-βAc-glo-SPA to create plasmids pTet-O-βAc-glo-ARE[5′-c-fos]-SPA and pTet-O-βAc-glo-ARE[GM-CSF]-SPA. These were used to prepare the second-generation plasmids that were used in this study. The region spanning the tetracycline operator and β-actin promoter elements of each of these plasmids was removed by digestion with NheI and NcoI, and replaced with the region spanning the tetracycline operator elements and minimal CMV promoter regions of pTREmyc. The latter was prepared by PCR amplification of pTREmyc using primers BM127 (5′-ATGCCAT-GGTGCTTAGACACGCG-3′) and BM128 (5′-CCGCTAGCTAGACACGGCCTTCTGTCG-3′). The final plasmids are designated pTet-CMV-βAc-glo-ARE[5′-c-fos]-SPA and pTet-CMV-βAc-glo-ARE[GM-CSF]-SPA.

**Western blotting**

Cells were washed twice with ice cold PBS and pelleted at maximum speed for 10 min in a microcentrifuge. Cell pellets were frozen on dry ice and thawed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% SDS, and a mixture of protease inhibitors [Roche Diagnostics]). Extracts were incubated on ice for 10 min and centrifuged for 5 min at maximum speed to recover the supernatant. Proteins were resolved by SDS/PAGE, transferred to nitrocellulose membrane, blocked, and incubated with rabbit anti–4E-T (1:1,000) (Dostie et al., 2000a), anti-Dcp1a (1:1,000), anti-Xp54 (1:2,000) (Smillie and Sommerville, 2002), and anti-eIF4A (1:1,000) (Kimball et al., 2003), and anti-HA (1:10,000) (Covance) antibodies or monoclonal anti-eIF4A (1:1,000) (Edery et al., 1998), anti-eIF4E (1:10) (Kimball et al., 2003), and anti-HA (1:1,000) (Covance) antibodies for 2 h at RT. Cells were washed extensively and incubated with Texas red or AlexaFluor 488 or 594 conjugated secondary antibody (1:200) (Molecular Probes) for 30 min. Nuclei were stained with Hoechst dye 33258 (Sigma-Aldrich). Images were taken from a Zeiss LSM 510 confocal microscope. Immunofluorescence analysis for each experiment was performed three times to five times, and the most representative results are displayed.

**Vaccinia virus infection, Far Western analysis, and translation assay**

HeLa cells (80% confluency) were infected with recombinant vaccinia virus for 1 h at 37°C (Fuerst et al., 1986). Cells were rinsed once with serum-free media and transfected with 5 μg each of the reporter plasmid (pGEM-LUC-POUieres-CAT) and pcDNA3–4E-T wt or mutant constructs using Lipofectin (15 μl/ml). RNA was resolved on a formaldehyde gel as described above and probed with 32P-labeled, random-primed DNA probe for determining to the manufacturer’s instructions. siRNA complexes were left overnight, and cells were processed 48 to 72 h later. 4E-T siRNA 5′-GAA-CAGAATTUUGCACCAGCA dTdT-3′; 4EIII inverted 5′-GCGACCAG-GCTTAAAGGTA dTdT-3′; 4EIII inverted 5′-CGUACCUGGUAAUGGUCU dTdT-3′.

**Transient transfections and immunofluorescence**

Transfections of DNA plasmids were performed by electroporation with the calcium phosphate method as described previously (Dostie et al., 2000a). HeLa cells were transfected at 50% confluency with 5 μg or 8 μg for GFP-4E-T of plasmid DNA, and were processed 36 h after transfection. For transfection of myc-EYFP-Me31B (Fig. 5 D), siRNA transfections were performed in 6-well plates as described above; after 24 h, 2 μg of myc-EYFP-Me31B was transfected by the calcium phosphate method. 48 h after siRNA transfection, cells were trypsinized into chamber slides and processed for immunofluorescence 12 h later. Immunofluorescence was performed as described previously (Dostie et al., 2000a). In brief, cells were plated onto Lab-Tek chamber slides (Nunc) 16 h before fixation. Cells were fixed with formaldehyde, permeabilized, blocked, and incubated with affinity-purified rabbit anti–4E-T (1:100) (Dostie et al., 2000a), affinity-purified rabbit anti-Dcp1a (1:200) (a gift from J. Lykke-Andersen, University of Colorado, Boulder, CO), and anti-Dcp2 (1:20) (Liu et al., 2004) antibodies, or monoclonal anti-eIF4A (1:100) (Edery et al., 1993), anti-eIF4E (1:10) (Kimball et al., 2003), and anti-HA (1:10,000) (Covance) antibodies for 2 h at RT. Cells were washed extensively and incubated with Texas red or AlexaFluor 488 or 594 conjugated secondary antibody (1:200) (Molecular Probes) for 30 min. Nuclei were stained with Hoechst dye 33258 (Sigma-Aldrich). Images were taken from a 63× objective of a Zeiss LSM 510 confocal microscope. Immunofluorescence analysis for each experiment was performed three times to five times, and the most representative results are displayed.

**Northern blotting**

Total RNA was isolated using a QIAGEN RNAeasy kit according to the manufacturer’s instructions. 5 μg of total RNA was separated on a 1.3% agarose/formaldehyde gel, transferred to a Hybond-N membrane (GE Healthcare), and probed with 32P-labeled, random-primer DNA probe to detect p21 (Cul et al., 2004) or 32P-labeled oligonucleotide, using terminal deoxynucleotidyl transferase (Invitrogen) to detect 18S RNA (Lal et al., 2004). For β-globin reporter assays, 4E-T or control siRNA was transfected into HeLa cells as described above. 36 h later, pTet-β-globin reporter plasmids and pTet Off (encoding for tTA, transactivator) were cotransfected. 12 h later, cells were split 1:6 and treated 16 to 20 h later with doxycycline (1 μg/ml). RNA was resolved on a formaldehyde gel as above and probed with 32P-labeled, random-primer DNA probe for detecting β-globin (Stoecklin et al., 2004) or GAPDH.

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**siRNA transfections**

siRNA annealed duplexes were purchased from Dharmaco and sequence data were listed. Below cells were transfected at 30% confluency in 6-well plates with siRNA (300 pmole final) using oligofectamine according to the manufacturer’s instructions. siRNA complexes were left overnight, and cells were processed 48 to 72 h later. 4E-T siRNA 5′-GAA-CAGAATTUUGCACCAGCA dTdT-3′; 4EIII inverted 5′-GCGACCAG-GCTTAAAGGTA dTdT-3′; 4EIII inverted 5′-CGUACCUGGUAAUGGUCU dTdT-3′.
anti-Dcp2, S. Kimball (The Pennsylvania State University College of Medicine, Hershey, PA) for monoclonal anti-tetR4E, H. Trachsel (University of Bern, Berne, Switzerland) for anti-tetR4A, and J. Sommerville [University of St. Andrews, St. Andrews, Scotland] and N. Standart (University of Cambridge, Cambridge, England) for anti-Xp54 antibodies. We are grateful to B. Seraphin [Centre National de la Recherche Scientifique, Gif-sur-Yvette, France] for GFP::Dcp2 plasmid, A. Nickaruma for myc-EYFP::U6-318 plasmid, and M. Gorospe [National Institutes of Health, Bethesda, MD] for p21 and IBS oligomers. We thank J. Garcia-Sanz for pTetQ-AcU3, and A.B. Shyu for pBBS+ARE- pls digram, respectively. We thank I. Gallouzi, F. Tremblay, and P. Cho for critical reading of the manuscript, and J. Labibette for technical support with the confocal microscopy. We are indebted to C. Uster and S. Perreault for assistance.

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