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Angiogenin cleaves tRNA and promotes stress-induced translational repression

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Stress-induced phosphorylation of eIF2α inhibits global protein synthesis to conserve energy for repair of stress-induced damage. Stress-induced translational arrest is observed in cells expressing a non-phosphorylatable eIF2α mutant (S51A), which indicates the existence of an alternative pathway of translational control. In this paper, we show that arsenite, heat shock, or ultraviolet irradiation promotes transfer RNA (tRNA) cleavage and accumulation of tRNA-derived, stress-induced small RNAs (tiRNAs). We show that angiogenin, a secreted ribonuclease, is required for stress-induced production of tiRNAs. Knockdown of angiogenin, but not related ribonucleases, inhibits arsenite-induced tiRNA production and translational arrest. In contrast, knockdown of the angiogenin inhibitor RNH1 enhances tiRNA production and promotes arsenite-induced translational arrest. Moreover, recombinant angiogenin, but not RNase 4 or RNase A, induces tiRNA production and inhibits protein synthesis in the absence of exogenous stress. Finally, transfection of angiogenin-induced tiRNAs promotes phospho-eIF2α–independent translational arrest. Our results introduce angiogenin and tiRNAs as components of a phospho-eIF2α–independent stress response program.

Introduction

The survival of mammalian cells exposed to adverse environmental conditions requires a radical reprogramming of protein translation (Yamasaki and Anderson, 2008). Stress-induced translational arrest of mRNAs encoding “housekeeping” proteins is triggered by a family of eIF2α kinases that reduce the availability of eIF2α–GTP–tRNA Met ternary complexes required for translation initiation (Anderson and Kedersha, 2008). Under these conditions, translation of a subset of mRNAs encoding upstream open reading frames (uORF; e.g., ATF4) is selectively enhanced, a consequence of uORF “read-through” (Lu et al., 2004). The reprogramming of protein translation is part of an integrated stress response that promotes the survival of cells subjected to adverse environmental conditions (Ron and Walter, 2007).

The finding that low-dose oxidative stress inhibits protein translation in cells expressing nonphosphorylatable eIF2α suggested the existence of a phospho-eIF2α–independent translation control pathway (McEwen et al., 2005). We hypothesized that stress-induced cleavage of tRNA, a phenomenon first described as a starvation response in Tetrahymena thermophila (Lee and Collins, 2005), and later observed in bacteria (Haiser et al., 2008), fungi (Jochl et al., 2008), and mammalian cells (Thompson et al., 2008), may contribute to stress-induced translational arrest. Sequence analysis reveals that tRNAs are cleaved in or near the anticodon loop. In both T. thermophila (Lee and Collins, 2005) and Saccharomyces cerevisiae (Thompson et al., 2008), many 3’ fragments possess “CCA” additions characteristic of mature tRNA. Moreover, these fragments lack tRNA introns, which indicates that they are not derived from pre-tRNAs. In most cases, the appearance of tRNA fragments is not accompanied by a significant depletion of mature tRNA, indicating that a small subset of the total tRNA population is cleaved during stress. In mammalian cells, analogous tRNA-derived fragments comprise a small subset of PIWI-associated RNAs (piRNAs), which suggests that tRNA anticodon cleavage may lead to the assembly of specific RNP complexes (Brennecke et al., 2007). It is possible that tRNA fragment-containing RNPs contribute to a phospho-eIF2α–independent translation control pathway.

Angiogenin is a secreted ribonuclease that was first identified as an angiogenic factor found in tumor cell–conditioned...
medium (Fett et al., 1985). The secretion of angiogenin is enhanced by hypoxia, which indicates that it may be a component of a stress response program (Hartmann et al., 1999; Nakamura et al., 2006). Angiogenin binds to receptors on the surface of endothelial cells that facilitate its internalization and transport to the nucleolus (Hu et al., 1997; Hatzi and Badet, 1999; Wiedlocha, 1999). Remarkably, promotion of new blood vessel growth is dependent on its ribonuclease activity (Shapiro and Vallee, 1987). Although the RNA targets required for angiogenesis are unknown, in vitro studies have shown that tRNAs are preferred targets (Saxena et al., 1992). Angiogenin also promotes ribosomal RNA transcription and cellular proliferation (Tsuji et al., 2005), which suggests that it has multiple functions. We have discovered that angiogenin is a stress-activated ribonuclease that cleaves tRNA and inhibits protein translation. Our results introduce angiogenin and tRNA-derived stress-induced RNAs (tiRNAs) as previously unappreciated components of the mammalian stress response.

Results and discussion

Extracts prepared from human U2OS cells exposed to arsenite-induced oxidative stress, heat shock, or UV irradiation were separated on a denaturing gel and developed with SYBR gold to visualize stress-induced small RNAs (Fig. 1 A). In stressed cells, two discrete bands corresponding to RNAs centered around 30 and 40 nucleotides were observed. Northern blotting using cDNA probes complementary to the 5′ end of tRNA^Met^ (Fig. 1 A, bottom, NB) and the 5′ and 3′ ends of tRNA^Met^, tRNA^Gly^, and tRNA^Pro^ (Fig. 1 B; 5S RNA is included as a loading control) confirms that the stress-induced RNAs are produced by tRNA cleavage. The size of these fragments requires that cleavage occur, as in *T. thermophila* (Lee and Collins, 2005), in or near the anticodon loop. These tiRNAs appear rapidly (within 20 min) in cells subjected to arsenite-induced oxidative stress, and persist for at least 11 h in cells allowed to recover from stress (Fig. S1 A). The phosphorylation and dephosphorylation of eIF2α over this time course provides a marker of stress and recovery from stress (Fig. S1 A). Arsenite-induced tiRNAs are observed in several different primate cell lines (Fig. S1 B). Importantly, tiRNAs are not observed in cells undergoing etoposide- or caffeine-induced apoptosis (Fig. S1 C), which indicates that tiRNA production is not a nonspecific consequence of cell death.

To determine the potential for tiRNAs to mediate phospho-eIF2α–independent translational arrest, we compared their

Figure 1. Stress-induced production of tiRNA. (A) U2OS cells treated with sodium arsenite (SA; 500 µM, 2 h), heat (42°C, 2 h), or UV irradiation (200 J/m², 12 h) were extracted with Trizol, and total RNA (10 µg) was separated on a 15% TBE-urea gel before processing with SYBR gold. The gel was also transferred to membrane and hybridized to a biotin probe complementary to the 5′ end of tRNA^Met^ (NB). (B) Northern blotting analysis of RNA extracted from U2OS cells cultured in the absence (−) or presence (+) of sodium arsenite (500 µM, 2 h). Blots were hybridized to cDNAs complementary to the 5′ or 3′ fragments of the indicated tRNAs (bottom) or 5S RNA as a loading control (top). (C) MEFs derived from wild type (wt) or eIF2α (S51A) mutant (mut) mice were cultured in the absence (−) or presence (SA) of sodium arsenite (500 µM) for the indicated times before Trizol extraction. tRNA and 5′ tRNA fragments were quantified by Northern blotting (top) and SYBR gold staining (middle). Phospho- and total eIF2α was quantified by immunoblotting (IB; bottom).
induction in mouse embryo fibroblasts (MEFs) derived from wild-type or eIF2α (S51A) mutant mice (Scheuner et al., 2001). The expression of mature tRNA<sup>Met</sup> is similar in wild-type and mutant (mut) cells in the absence or presence of arsenite (Fig. 1 C, SA). In both wild-type and mutant MEFs, tiRNAs are much less abundant than tRNAs. Although densitometric analysis may be misleading because of the relative overexpression of tRNA<sup>Met</sup>, the calculated ratios of tiRNA<sup>Met</sup>/tRNA<sup>Met</sup> are always <0.1, which indicates that depletion of tRNA is unlikely to contribute to the functional effects of tRNA cleavage. Interestingly, the induction of tiRNA<sup>Met</sup> is significantly greater in mutant cells compared with wild-type cells, indicating that phospho-eIF2α is not required for, and may inhibit, tRNA production. We also quantified the production of tiRNAs in U2OS cells treated with control or heme-regulated initiation factor 2-α kinase (HRI)-specific siRNAs (Fig. S2). Knockdown of HRI, the eIF2α kinase activated by arsenite, increases the arsenite-induced production of tiRNAs. Collectively, these results indicate that the induction of tiRNAs does not require phospho-eIF2α. Moreover, phospho-eIF2α may suppress the induction of tiRNA.

Ribonucleases that target the anticodon loop of tRNA are found in both prokaryotes and eukaryotes (Ardelt et al., 1991; Kaufmann, 2000; Suhasini and Sirdeshmukh, 2006). In Xenopus oocytes, a tRNA anticodon nuclease designated onclease has been found to inhibit protein synthesis and promote the death of selected tumor cells (Ardelt et al., 1991; Suhasini and Sirdeshmukh, 2006). The toxic effects of onclease are observed at doses that do not markedly deplete cellular tRNAs, which suggests that activation of the ribotoxic stress response may contribute to its toxic effects (Iordanov et al., 2000).

Angiogenin is an onclease-related ribonuclease that selectively cleaves tRNA in mammalian cells (Saxena et al., 1992). To determine whether angiogenin is required for the stress-induced production of tiRNA, we used siRNA to knock down angiogenin expression before quantifying arsenite-induced tiRNA production and stress-induced translational repression. Transfection of a Dharmacon SMART pool targeting angiogenin reduces the expression of angiogenin mRNA (quantified using quantitative RT-PCR) to 63 ± 8% (n = 5) of the control level. Under these conditions, the arsenite-induced production of tiRNA is reduced to 55 ± 6% (n = 3) of the control level (Fig. 2 A, lane 3; representative of three independent experiments). This result was confirmed using an angiogenin-specific siRNA that more efficiently reduces angiogenin expression (Tsuji et al., 2005). This reagent reduces the expression of angiogenin mRNA (quantified using quantitative RT-PCR) to 27% ± 12 (n = 3) of the control level, and production of tiRNAs to 36 ± 8% (n = 3) of the control level (Fig. 2 A, lane 4; representative of three independent experiments). To determine the effect of angiogenin on stress-induced translational repression, U2OS cells were treated with the indicated siRNAs, then pulsed with [35S]methionine-containing medium in the absence or presence and pulsed with [35S]methionine-containing medium for 60 min before protein extraction. [35S]Methionine incorporation (mean ± SD, n = 3–5) is normalized to that observed in cells treated with control siRNA (designated 100%). * P = 0.02 (n = 3); **, P = 0.04 (n = 5).
Targeted knockdown of RNH1 induces the production of tiRNAs in the absence or presence of arsenite-induced oxidative stress (Fig. 3 A, lanes 2 and 4, respectively). This result suggests that angiogenin is constitutively expressed but held in an inactive state by RNH1. In cells subjected to arsenite-induced oxidative stress, knockdown of RNH1 enhances tiRNA production and promotes stress-induced translational silencing (Fig. 3 B, compare lanes 3 and 4). In unstressed cells, tiRNAs induced by RNH1 knockdown do not inhibit protein synthesis under the assayed conditions (Fig. 3 B, compare lanes 1 and 2). This may be due to a requirement for stress- or secreted angiogenin–induced cofactors. Further experiments will be needed to clarify this point.

Because angiogenin is a stress-induced secreted protein that is taken up by adjacent cells, we tested the ability of purified recombinant angiogenin to induce the production of tiRNAs in U2OS cells. Wild-type angiogenin, but not an inactive mutant that has been implicated in the pathogenesis of amyotrophic lateral sclerosis (P112L; Fig 4 A, MUT; Wu et al., 2007), induces the production of tiRNA in U2OS cells (Fig. 4 A).
The angiogenin-related ribonucleases RNase 4 and RNase A do not induce the production of tiRNA under these conditions (Fig. 4 B). Wild-type angiogenin, but not an inactive mutant (ANG-H13A; Shapiro et al., 1986), or RNase 4, significantly inhibits global protein synthesis in U2OS cells (Fig. 4 C). These results are consistent with a role for angiogenin in both stress-induced tRNA cleavage and stress-induced translational arrest.

Endogenous tiRNAs corresponding to 5’ and 3’ tRNA fragments were purified from angiogenin-treated cells and transfected into U2OS cells. Pulse labeling with [35S]methionine reveals that 5’ tiRNAs, but not 3’ tiRNAs or synthetic control RNAs (sequences corresponding to PIWI-associated RNAs; see Materials and methods) significantly inhibit protein synthesis (Fig. 5 A). The combination of 5’ and 3’ tiRNAs also inhibits translation, indicating that 3’ tiRNA does not inhibit the activity of 5’ tiRNA. Importantly, the ratio of transfected tiRNA/tRNA (Fig. 5 B) is similar to the ratio of endogenous tiRNA/tRNA observed in arsenite-treated cells (Fig. 2 A). Similar results were obtained when 5’ and 3’ tiRNAs were transfected into wild-type (Fig. 5 C, SS) and S51A mutant MEFs (Fig. 5 C, AA), which indicates that inhibition of protein synthesis does not require phosphorylation of eIF2α, ruling out a primary role for PKR in this process. Autoradiographic analysis of 35S-labeled proteins reveals that 5’ tiRNAs inhibit global protein synthesis (Fig. 5 D).

Angiogenin is a secreted protein that functions in the acute phase response induced by inflammatory stimuli (Olson et al., 1998). We have shown that: (1) recombinant angiogenin induces the production of tiRNAs in U2OS cells (while this manuscript was under review, similar results were reported by Fu et al. [2009]), (2) recombinant angiogenin inhibits protein synthesis in U2OS cells, (3) knockdown of angiogenin inhibits arsenite-induced tiRNA production and translational repression, (4) knockdown of RNH1 enhances tiRNA production and promotes arsenite-induced translational repression, and (5) transfection of purified, endogenous 5’ but not 3’ tiRNAs inhibits protein synthesis in U2OS cells as well as wild-type and S51A mutant MEFs. Collectively, these results strongly implicate angiogenin and tiRNAs in a process of stress-induced translational repression.

The finding that tRNA fragments possess posttranscriptionally added “CCA” residues and lack leaders, trailers, and introns suggests that tiRNAs are derived from mature tRNAs (Lee and Collins, 2005; Thompson et al., 2008; Fu et al., 2009). We have found that neomycin, a drug that prevents recombinant angiogenin from entering the nucleus, has no effect on angiogenin-induced tRNA cleavage, which suggests that angiogenin or presence of sodium arsenite (SA; lane 2; 500 µM, 1 h), recombinant wild-type angiogenin (WT; 1 µg/ml, lane 3), or recombinant mutant angiogenin [MUT, 1 µg/ml, lane 4] for 1 h before Trizol extraction, separation by 15% TBE-urea gel, and CYBR gold staining. The tRNA positions are indicated on the left. (B) U2OS cells were treated with the indicated ribonucleases (1 µg/ml) for 1 h before processing as described in A. (C) U2OS cells were treated with the indicated ribonucleases (1 µg/ml) for 30 min in the presence of [35S]methionine-containing medium before protein extraction. 35S counts in cells cultured in media alone (Cont) were normalized to 100%. Results are the means ± SD (n = 3). *P = 0.02.
likely to be 5' monophosphates. In contrast, the 5' ends of 3'-tiRNAs are likely to be hydroxyl groups. As miRNAs and piRNAs have 5' monophosphates, this modification may promote the stability and function of small RNAs. The 3' ends of 5'-tiRNAs are likely to be 2', 3' cyclic phosphates, as angiogenin cleavage leaves this moiety at the 3' ends of cleaved RNAs (Rybak and Vallee, 1988). This cyclic phosphate residue may be resolved to 2' phosphate or 3' phosphate groups. Whether this moiety is
Materials and methods

Cell culture and medium

U2OS cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum (Sigma-Aldrich), 100 U/ml antibiotics (penicillin), and 100 µg/ml streptomycin. Lipofectamine 2000 (Invitrogen) and Opti-Mem (Invitrogen) were used for transfection of tiRNAs and siRNAs. The wild-type (SS) and S51A knock-in (AA) MEFs, a gift from D. Scheuner and R. Kaufman (University of Michigan Medical Center, Ann Arbor, MI), were cultured in DMEM with 10% fetal calf serum and antibiotics. For stress induction, the indicated doses of sodium arsenite (Sigma-Aldrich) were added in medium. The cells were washed with PBS twice before UV irradiation (UV cross-linker FB-UVXL-1000; Fisher Biotech). Heat shock was achieved by incubating cells in a 42°C oven. U2OS cells were treated with recombinant wild-type or mutant angiogenin (Wu et al., 2007), RNA4 4 purified using the same methods used for angiogenin), or RNase A 0.5 µg/ml; QIAGEN) for 1 h before processing for quantification of tiRNAs.

RNA analysis

Total RNA was extracted by using Trizol (Invitrogen). RNA (10 µg per well) was analyzed using TBS-urea gels (Invitrogen) or 1.1% agarose/2% formaldehyde MOPS gels, transferred to Nytran Supercharge membranes (Schleicher and Schuell), and hybridized overnight at 50° with digoxigenin (DIG)-labeled RNA probes in DIG Easy Hyb solution (Roche). After washing at 60° with 2× SSC/0.1% SDS (10 min) and 0.5× SSC/0.1% SDS, the membranes were washed again with 5× SDS-digoxigenin bovine serum (Thermo Fisher Scientific) for 30–60 min, replaced with fresh labeling medium containing ~150–250 µCi of [35S]methionine per well (EasyTag EXPRESS 35S Protein Labeling Mix; PerkinElmer) and incubated for 30 min. Recombinant ribonucleases or arsenite was added together with the 35S-labeled mix and incubated for 30 min or 60 min, respectively, before processing. After washing with PBS twice, cells were harvested in 400 µl of lysis buffer (2% SDS/20 mM Hepes, pH 7.4) and sonicated, and the protein was precipitated by the addition of 60% acetone. The proteins were resuspended in lysis buffer, and 10 µl of each sample in Ecoscint H (National Diagnostics) was counted using a liquid scintillation counter (Beckman Coulter). Protein concentration was determined by Protein Assay BCA Protein Assay kit (Pierce).

Antibodies and reagents

Antibodies against phospho-eIF4E or total eIF4E were obtained from Assay Designs or Santa Cruz Biotechnology, Inc., respectively. Polyclonal rabbit anti-RN1H1 was obtained from Proteintech Group, Inc.

tiRNA isolation and transfection

U2OS cells (6.0 × 106) were treated with 0.5 µg/ml recombinant angiogenin or 500 µM sodium arsenite for 90 min before extraction with Trizol. 2 µg of total RNA was separated using four sets of 15% TBE-urea acrylamide gels. Gel fractions containing 5’ tiRNA and 3’ tiRNA visualized using SYBR gold nucleic acid gel stain (Invitrogen) were crushed and soaked in 20 µl of nuclease-free 1 M NaCl with rocking at 4°C overnight. After centrifugation, linear acrylamide (Applied Biosystems) and ethanol (to 60%) were added to each supernatant. The RNA mixtures were filtered through a MEGAclear filter cartridge (Applied Biosystems). After washing the filter with 4 ml of 80% ethanol, the MEGAclean filter cartridges were washed again with 500 µl of 80% ethanol. After removing excess ethanol, the bound tiRNAs were eluted by 80 µl of heated (95°C) elution solution. U2OS cells or MEFs were transfected with 5’ or 3’ tiRNAs (1 µM) using Lipofectamine 2000. After 6 h, washed cells were metabolically labeled as described in “Metabolic labeling.”

siRNA sequences

The RNA sequences were obtained from the RNA World Website (http://www.imb-jena.de/RNA.html) at the Leibniz Institute for Age Research, Fritz Lipmann Institute.

siRNA treatment

For angiogenin knockdown, HRI, RN1H1, RNaseL, ELAC2, and RNase T2 siGENOME SMART pools for each molecule were purchased from Thermo Fisher Scientific. The individual angiogenin siRNA sequence was 5’-GAG UUGCUUAUUCUUAGGUGGUU-3’. U2OS cells were transfected with 40 nM of siRNA using Lipofectamine 2000 12 h after plating cells. On the next day, the cells were replated, and a second siRNA transfection was performed, after which cells were cultured for 24 h. Total RNA was isolated using Trizol after a 90-min incubation with 500 µM sodium arsenite.

RNA oligos

ctrRNA1 (pR58620, control RNA): 5’-UGUGAGAGACGUGAGGCAGCAGCAGGUCUC-3’; ctrRNA2 (pR006650, control RNA): 5’-UGAGGGGUGUUGGUGUCUCUAUUCCUCUC-3’; ctrRNA3 (pR016792, control RNA): 5’-CCUCUCAAGUGUGGGAUCAAUCGUCCUC-3’. For angiogenin knockdown, HRI, RNH1, RNaseL, ELAC2, and RNase T2

DNA oligos


Angiogenin-induced tiRNAs inhibit protein translation • Yamashita et al.

Further modified to produce the protective 2’-O-methyl groups found at the termini of miRNAs and piRNAs (Horwich et al., 2007) is an important question for future research. In contrast, the 3’ ends of 3’ tiRNAs, like tRNAs, are likely to be hydroxyl groups. Thus, the nature of the 5’ and 3’ ends of these tRNA fragments may be an important determinant of their functional potential.

Because stress-induced reprogramming of protein translation can help cells survive adverse environmental conditions, secreted angiogenin may activate an “infectious” stress response program that allows stressed cells to warn their brethren of approaching noxious stimuli. This may occur at the organinal level by secretion of angiogenin from the liver (Olson et al., 1998), or at the tissue level by secretion of angiogenin from stressed cells within peripheral tissues. The local and systemic actions of angiogenin would be consistent with its expression from an upstream promoter that is expressed in all tissues and a downstream promoter that is expressed in liver cells (Dyer and Rosenberg, 2005). Just as interferons are virus-induced factors that protect adjacent or distant cells from virus infection, angiogenin may be a stress-induced factor that protects adjacent or distant cells from the deleterious effects of environmental stress.

Angiogenin has been also implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS), a neurological disease caused by the death of motor neurons. Mutations in angiogenin are found in a small subset of patients with both familial and sporadic ALS (Greenway et al., 2006; Wu et al., 2007). ALS-associated angiogenin missense mutations have reduced ribonuclease and angiogenic activity (Greenway et al., 2006; Wu et al., 2007). It is therefore possible that the survival of motor neurons requires the angiogenin-induced stress response program.

Metabolic labeling

In in vivo experiments, control RNA or tiRNAs were transfected into U2OS cells or MEFs in 24-well plates, and cultured for the indicated times. The cells were incubated with labeling medium (DMEM without L-glutamine, sodium pyruvate, L-methionine, or L-cysteine [Invitrogen], supplemented with 5% diazylated fetal bovine serum [Thermo Fisher Scientific]) for 30–60 min, replaced with fresh labeling medium containing ~150–250 µCi of [35S]methionine per well (EasyTag EXPRESS 35S Protein Labeling Mix; PerkinElmer) and incubated for 30 min. Recombinant ribonucleases or arsenite was added together with the 35S-labeled mix and incubated for 30 min or 60 min, respectively, before processing. After washing with PBS twice, cells were harvested in 400 µl of lysis buffer (2% SDS/20 mM Hepes, pH 7.4) and sonicated, and the protein was precipitated by the addition of 60% acetone. The proteins were resuspended in lysis buffer, and 10 µl of each sample in Ecoscint H (National Diagnostics) was counted using a liquid scintillation counter (Beckman Coulter). Protein concentration was determined by Protein Assay BCA Protein Assay kit (Pierce).
References


