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MINI-REVIEW

Apoptotic regulation and tRNA

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ABSTRACT

Apoptotic regulation is critical to organismal homeostasis and protection against many human disease processes such as cancer. Significant research efforts over the past several decades have illuminated many signaling molecules and effecter proteins responsible for this form of programmed cell death. Recent evidence suggests that transfer RNA (tRNA) regulates apoptotic sensitivity at the level of cytochrome *c*-mediated apoptosome formation. This finding unexpectedly places tRNA at the nexus of cellular biosynthesis and survival. Here we review the current understanding of both the apoptotic machinery and tRNA biology. We describe the evidence linking tRNA and cytochrome *c* in depth, and speculate on the implications of this link in cell biology.

KEYWORDS apoptotic regulation, tRNA, cytochrome *c*

CYTOCHROME *C* AND THE INTRINSIC APOPTOTIC PATHWAY

Apoptosis is the major process through which unnecessary, damaged, and harmful cells are eliminated. Multi-cellular development, homeostasis, immunity and protection against cancer all require intricate regulation of programmed cell death (Thompson, 1995; Vaux and Korsmeyer, 1999). Two major apoptotic routes exist in mammalian cells: the extrinsic and intrinsic pathways. The final common end point of both pathways is activation of the proteolytic caspase (cysteine-dependent aspartate specific protease) cascade. This causes cleavage of numerous cellular targets resulting in cellular shrinkage, fragmentation, membrane blebbing and nuclear condensation and termination of cell life (Chang and Yang, 2000; Li and Yuan, 2008). The extrinsic pathway is activated by engaging death receptors such as Fas and TRAIL receptors with their cognate ligands (Ashkenazi and Dixit, 1998). This leads to the formation of the membrane-bound death inducing signaling complex (DISC), which recruits the initiator procaspase-8 by the adaptor protein FADD. In the DISC, procaspse-8 is activated by oligomerization (Yang et al., 1998; Chang et al., 2003). The intrinsic pathway responds to "intracellular" cues such as DNA damage, oncogene activation, nutrient deprivation, and lineage information. Intrinsic apoptotic signals converge to cause mitochondrial outer membrane permeabilization (MOMP). This leads to the release of cytochrome c, an essential electron transfer chain component, from mitochondria to cytosol (Wang, 2001). The discovery of the role of cytochrome *c* in apoptosis (Liu et al., 1996) came as a shock to a field that regarded apoptosis as being carried out by deadly signaling molecules. It casts light on an ingenious design during evolution that has endowed a potent destructive power to a molecule so essential for life, and gives a striking physical form for the philosophic phrase that life and death are inseparable.

In the cytosol, cytochrome *c* is a ligand for Apaf-1 (the apoptotic protease activating factor-1) (Zou et al., 1997). Apaf-1 binds to dATP or ATP in an inactive state, and cytochrome *c* binding stimulates the intrinsic (d)ATPase activity of Apaf-1 leading to the hydrolysis of (d)ATP to (d)ADP. Subsequently, (d)ADP is exchanged with a free (d)ATP molecule in the cytosol (Kim et al., 2005). Then, with the assistance of at least three proteins: HSP70, cellular apoptosis susceptibility protein (CAS), and putative HLA-DR-associated protein 1 (PHAP1), Apaf-1 assemblies into a heptameric, wheel-like structure known as the apoptosome (Acehan et al., 2002; Kim et al., 2008). Oligomerized Apaf-1 recruits caspase-9 through an interaction involving the caspase recruitment domain (CARD) on both proteins. Caspase-9 is subsequently activated by oligomerization (Boatright et al., 2003).

PHYSIOLOGIC AND PATHOLOGICAL REGULATION OF CYTOCHROME *C*-MEDIATED CASPASE-9 ACTIVATION

Regulation of cytochrome *c*-mediated caspase-9 activation occurs both before and after cytochrome *c* release. Prior to cytochrome *c* release, Bcl-2 family proteins are major regulators of the apoptotic switch. These proteins, which contain up to four Bcl-2 homolog (BH1 to BH4) domains, are divided into an anti-apoptotic subfamily (e.g., Bcl-2, Bcl-XL, and Mcl-1) and two pro-apoptotic subfamilies: multiple BH domain-containing proteins (e.g., Bax and Bak) and BH3-only proteins (e.g., Bid) (Adams and Cory, 1998). BH3-only proteins act furthest upstream and are activated by various mechanisms including increased transcription, phosphorylation, and proteolytic processing (Huang and Strasser, 2000). While the precise mechanisms are unclear, BH3-only proteins promote cytochrome *c* release either directly by stimulating multiple BH domain-containing proteins to form pores on the mitochondrial outer membrane or indirectly by counteracting anti-apoptotic Bcl-2 proteins (Chipuk and Green, 2008).

A range of cellular factors regulates apoptosome formation after cytochrome *c* release. The physiologic concentrations of potassium and calcium suppress apoptosome formation, the latter by preventing nucleotide exchange by Apaf-1 (Cain et al., 2001; Bao et al., 2007). HSP70 and HSP90 have been demonstrated to interact with Apaf-1 and inhibit interaction between Apaf-1 and caspase-9 (Beere et al., 2000; Bruey et al., 2000; Pandey et al., 2000; Saleh et al., 2000), while HSP27 binds to cytochrome *c* and blocks its interaction with Apaf-1 (Bruey et al., 2000). The oncoprotein prothymosin-α (Pro-T) also impairs apoptosome assembly although the mechanism is undefined (Jiang et al., 2003). Furthermore, although low levels of dATP or ATP promote apoptosome formation, high levels of these nucleotides inhibit apoptosome by directly binding to cytochrome *c* (Chandra et al., 2006).

Post-translational modification of cytochrome *c* is critical for apoptosome formation. Newly synthesized cytochrome *c* is unable to activate Apaf-1 until it undergoes heme attachment concurrent with its import into mitochondria (Martin and Fearnhead, 2002). Additionally, oxidized but not reduced cytochrome *c* has been found to activate caspases (Saleh et al., 2000). In the physiological setting, intracellular glutathione (GSH), generated as a result of glucose metabolism by the pentose phosphate pathway, is necessary for maintaining cytochrome *c* in a reduced form and abrogating its pro-apoptotic function (Vaughn and Deshmukh, 2008).

Cellular life and death decisions can be changed even after apoptosome formation. Cytosolic cytochrome *c*-mediated caspase activation is directly inhibited by the inhibitor of apoptosis (IAP) proteins. IAPs were originally identified in baculoviruses, and homologs are present throughout the tree of life. Each IAP protein contains at least one but often two to three characteristic zinc binding BIR sequences (baculovirus IAP repeats) (Salvesen and Duckett, 2002). In mammalian cells, X-linked IAP (XIAP) is a potent caspase inhibitor. XIAP binds to partially processed caspase-9 and blocks its activity and further activation. XIAP also inhibits caspase-3 and caspase-7 (Salvesen and Duckett, 2002).

The intrinsic apoptotic pathway is frequently inactivated in tumor cells by a variety of mechanisms. Several of the more prominent examples are described here. The tumor suppressor p53 is probably the most commonly mutated gene in human cancer. Wild type p53 activates transcription of Bax and BH-3-only proteins (e.g., Puma, Noxa, and Bid), while also acting as a BH3-only protein to induce cytochrome *c* release independently of transcription. Both functions are disabled by most, if not all, tumor-associated p53 mutations (Vousden and Lane, 2007). The Bcl-2 family is also widely dysregulated in tumors. The founding member of this group, Bcl-2, was identified as a highly expressed oncogene in human follicular B cell lymphomas due to a chromosomal translocation that places Bcl-2 adjacent to the *IgG* heavy chain locus (Adams and Cory, 1998). Mcl-1 is another well-known anti-apoptotic Bcl-2 protein. This protein is rapidly degraded in normal cells, but its stability is increased in some tumor cells (Schwickart et al., 2010). Apaf-1 expression is decreased in some melanoma cells by hyper-methylation of the *Apaf-1* promoter (Soengas et al., 2001). Another common mutation in tumor cells is the highly active PI-3K/Akt pathway. One target for this pathway is the BH3-only protein Bad, which upon phosphorylation forms a complex with 14-3-3 and loses its pro-apoptotic activity (Datta et al., 1997).

tRNA, and ribonucleic acid in general, is a recently identified and unique player in apoptotic regulation (Mei et al., 2010). Changes in the levels of tRNA and other RNAs are ubiquitous in oncogenic settings (Ruggero and Pandolfi, 2003; White, 2005). This raises the attractive possibility that RNAs play a pivotal role in the regulation of apoptosis.

CANONICAL tRNA FUNCTION: THE ADAPTOR FOR PROTEIN SYNTHESIS

tRNAs arose early in evolution as the adaptors in the translation of genetic information into protein sequences. All tRNA molecules fold into a conserved cloverleaf secondary structure through regions of internal self-complementarity. A set of conserved nucleotides then facilitates the formation of a compact L-shaped tertiary structure (Fig. 1). The folding of the L shape involves coaxial stacking of the acceptor stem with the T stem-loop to form the top arm of the L, and coaxial stacking of the dihydrouridine stem (D-stem) with the anticodon stem-loop to form the vertical arm of the L. tRNAs are differentiated from each other according to the attached amino acid at the 3' end, which matches the anticodon triplet on the vertical arm. This match of an amino acid with a trinucleotide sequence in tRNA is the underlying basis of the

genetic code. It is through this match that aminoacyl-tRNAs deliver amino acids to specific codon positions on the ribosome through base pairing interactions with tRNA anticodons, thus enabling decoding of mRNA sequences into amino acid sequences. The degeneracy of the genetic code, whereby more than one triplet codons correspond to a given amino acid, is achieved by having multiple tRNAs (known as isoacceptors) with the same amino acid specificity but with distinct anticodons.

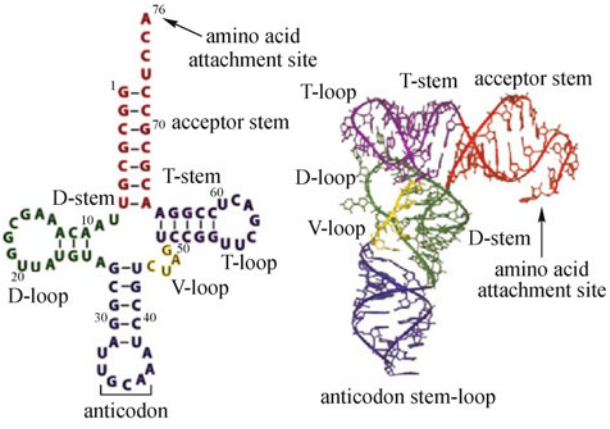

Figure 1. Sequence and structure of *E. coli* tRNA^{Cys}: (left) the cloverleaf secondary structure and (right) the L-shaped tertiary structure.

Many enzymes required for the tRNA adaptor function are both highly conserved and essential for growth. For example, all mature tRNAs possess the CCA sequence at the 3′ end as the obligate site for aminoacylation and for stable interaction with the ribosome (Fig. 1). This sequence is not encoded in human genomic DNA and must be added post-transcriptionally by the CCA-adding enzyme, which exists in all living organisms. The human CCA enzyme shares considerable sequence and structural homology with its bacterial counterparts (Yue et al., 1996). Aminoacylation of tRNA is catalyzed by aminoacyl-tRNA synthetases (aaRSs); there are 20 such enzymes in mammalian cells, one for each canonical amino acid, and these enzymes are highly conserved throughout their respective amino acid families.

The human mitochondrial genome encodes a separate set of tRNAs specifically for protein synthesis in the organelle. Although mitochondrial tRNAs generally have lower GC content and shorter stem-loop regions compared to their cytoplasmic counterparts, they still appear to fold into the same conserved L-shaped tertiary structure (de Bruijn and Klug, 1983; Watanabe et al., 1994). Importantly, protein enzymes that operate on mitochondrial tRNAs for the adaptor functions in the organelle, such as the mitochondrial CCA enzyme, and the 20 mitochondrial aminoacyl-tRNA synthetases are encoded by the nuclear genome. These nuclear-encoded mitochondrial enzymes nonetheless share high sequence homology with their cytoplasmic counterparts. The strong conservation of protein components that enable and promote the tRNA adaptor function emphasizes the importance of this role in biology.

NON-CANONICAL FUNCTIONS OF tRNA

Several recent examples suggest that tRNAs have important extra-translational functions. First, replication of the RNA genome of human immunodeficiency virus 1 (HIV-1) requires the CCA sequence of the human host $tRNA_3^{Lys}$ as the primer for initiation of the replication cycle (reviewed in Kleiman et al., 2010). The specific $tRNA_3^{Lys}$, together with the other two $tRNA^{Lys}$ isoacceptors and the aminoacylation enzyme lysyl-tRNA synthetase (LysRS), are also required for packaging of the virus. The molecular interactions that direct the assembly of the $tRNA^{Lys}$/LysRS packaging complex suggest the potential for developing new anti-viral agents. Second, while the translation initiator $tRNA_i^{Met}$ plays a crucial role in the initiation of protein synthesis, it also has the ability to act as a pre-mRNA splicing regulator in a manner independent of its role in protein synthesis. Specifically, alternative splicing events resulting from AUG codon mutations are suppressible by initiator $tRNA_i^{Met}$ variants harboring anticodon mutations that match the AUG mutations (Kamhi et al., 2010). This mechanism of regulation of splicing appears to play a role in quality control of splicing in the cell nucleus, preventing the generation of premature termination codons. Third, tRNA is a sensor of stress and nutrient deprivation that responds to these and other adverse situations by translocating in a retrograde fashion from the cytosol to the nucleus (Shaheen et al., 2007). This retrograde movement reduces tRNA availability in the cytosol, perhaps minimizing energy expenditure from protein synthesis. Uncharged tRNAs also accumulate in nutrient deprivation conditions and are efficient activators of the GCN2 kinase pathway (Hinnebusch, 2005). Activated GCN2 kinase phosphorylates the initiation factor eIF2a, causing inactivation of the initiation factor eIF2B, and repression of GCN4, an activator of general protein synthesis. This reduces the overall rate of protein synthesis, limiting amino acid consumption while allowing the cell to translate appropriate amino acid biosynthesis and stress-response genes.

Stress conditions also result in cleavage of tRNAs near anticodon sequences, resulting in tRNA half molecules (Thompson et al., 2008; Thompson and Parker, 2009a, b). These tRNA fragments have been identified in a wide variety of stress conditions, particularly during amino acid starvation or oxidative stress. Stress-induced tRNA cleavage may be catalyzed by a Dicer-dependent complex (Cole et al., 2009), or by the enzyme angiogenin (Yamasaki et al., 2009), a member of the RNase A family. Angionenin is typically sequestered in the nuclear compartment but is released into the cytosol during stress (Yamasaki et al., 2009). Production

of tRNA fragments does not significantly deplete total tRNA levels, suggesting fragment generation has a function beyond depletion of cellular tRNA pools. One hypothesis is that tRNA fragments may inhibit global translation by forming repression complexes to block the initiation or elongation steps of protein synthesis (Yamasaki et al., 2009), or to affect the degradation or repression of specific mRNAs by recruiting tRNA processing enzymes to mRNAs (Elbarbary et al., 2009a, b). One tRNA fragment in particular has been implicated in proliferation of a broad range of cancer cells (Lee et al., 2009), indicating a role in tumor progression.

THE CYTOCHROME *C* AND tRNA CONNECTION

We began examining possible roles for RNA in regulating apoptosis many years ago. A puzzling observation was that up to 1 mM concentration of dATP is needed to induce caspase-9 activation in cell lysates while the intracellular concentration of dATP is only in 10 μM range (Liu et al., 1996; Mesner et al., 1999). One possible explanation for this was that an inhibitor was present in the cell lysates that decreased the effectiveness of dATP. It seemed possible to us that RNA, essentially a polymer of nucleoside monophosphates, might have an inhibitory effect. This was supported by the observations that RNase treatment of S100 HeLa and Jurkat cell lysates strongly increased caspase-9 activation, and that addition of total cellular RNA to either S100 extracts or a reconstituted caspase-9 activation system potently blocked caspase-9 activation. These results implicate an inhibitory role of RNA in the activation of caspase-9 (Mei et al., 2010).

Systematic examination of the steps leading to caspase-9 cleavage identified cytochrome *c* as the target of the RNA inhibitor. Analysis of cytochrome *c*-associated species found that it was tRNA that binds specifically to cytochrome *c* both *in vitro* and in cells. The relevance of this finding in living cells was demonstrated by the ability of microinjected tRNA to inhibit cytochrome *c*-induced apoptosis, and by the ability of a tRNA-specific RNase to enhance both apoptosis and caspase-9 activation (Mei et al., 2010). Taken together, these results show that tRNA binds to cytochrome *c* and inhibits the formation of the apoptosome (Fig. 2).

This finding raises intriguing questions regarding the generality of a role of tRNA in apoptotic and other protein functions. Below we discuss briefly the implications of this finding in the context of cell death, proliferation, and oncogenic transformation.

ARE CYTOCHROME *C* AND tRNA LIVING AN AGE-OLD ROMANCE?

Both cytochrome *c* and tRNA are ancient molecules. tRNA is present in all known forms of life, and its secondary and tertiary structures are highly conserved across species and organelles (see above). Cytochrome *c* is present in most eukaryotes that have a mitochondrial electron transport chain. Cytochrome *c* from any species usually cross-reacts with cytochrome *c*-oxidase enzymes from another species, underscoring the conservation of cytochrome *c* function throughout evolution. We speculate that the cytochrome *c*:tRNA interaction is similarly evolutionarily ancient. Given the small size (~100 aa) and highly positively charged nature of cytochrome *c*, its preferential binding to tRNA relative to other RNAs is striking. The structural nature of this association should be highly interesting.

Suppression of the cytochrome *c*'s pro-apoptotic function by tRNA links protein synthesis to apoptotic susceptibility. Cells with high translation rates are often rapidly proliferating, and increased apoptotic resistance may prevent futile cycles of cellular proliferation and death. The cytochrome *c*:tRNA connection may also contribute to apoptotic resistance of tumor cells, which is supported by multiple lines of evidence. Human cytosolic tRNAs are transcribed by RNA polymerase III (pol III) (Lowe and Eddy, 1997; White, 2004). The critical tumor suppressors p53 and Rb directly bind and inhibit pol III, while c-Myc and Ras activate pol III transcription (Larminie et al., 1999; Crighton et al., 2003; Gomez-Roman et al., 2006). Increased biosynthesis of tRNA is required for proliferation and likely represents an obligatory step in tumorigenesis (White, 2005; Marshall et al., 2008). Indeed, tRNA is highly expressed in tumor cells (White, 2005).

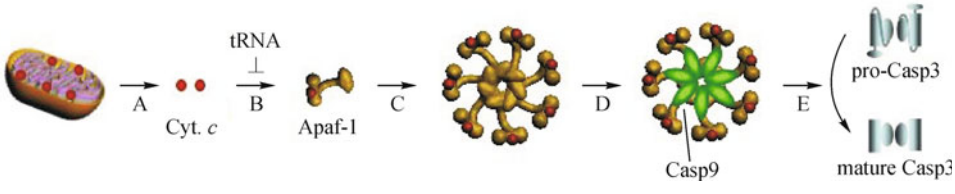

Figure 2. Cytochrome *c*-mediated caspase activation. Intracellular apoptotic stimuli provoke the release of cytochrome *c* (cyt. *c*) from mitochondria to the cytosol (A), where it binds to Apaf-1 (B) promoting the assembly of Apaf-1 into the heptameric apoptosome (C). The apoptosome recruits and oligomerizes the precursors of caspase-9 (Casp9), leading to its auto-proteolytic processing (D). Mature caspase-9 then activates procaspase-3 (pro-Casp3) through trans-cleavage (E). tRNA binds to cytochrome *c* and prevents its interaction with Apaf-1.

For example, breast cancer cells have 4–5 fold increased median nuclear tRNA expression and 5–20 fold increased median mitochondrial tRNA expression (Pavon-Eternod et al., 2009). Additionally, several RNase drugs have shown specific anti-tumor activity that is dependent on their catalytic ability (Costanzi et al., 2005). Onconase/Ranpirnase is the furthest developed and has reached phase III clinical trials for the lung tumor mesothelioma, and is in phase II trials for several other cancers (Costanzi et al., 2005). Onconase preferentially cleaves tRNA in a manner that correlates with apoptotic sensitivity (Iordanov et al., 2000; Saxena et al., 2002; Suhasini and Sirdeshmukh, 2006). As a potential chemotherapeutic agent, Onconase has attractive attributes, including low systemic toxicity and p53-independent killing. We believe that a better understanding of the role of tRNA in apoptotic resistance should provide a rational basis for the use and improvement of tRNA-based tumor therapy.

Relative to its role in electron transport, the ability of cytochrome *c* to initiate apoptosis is a relatively recent evolutionary phenomenon. For example, cytochrome *c* from *C. elegans* does not induce apoptosome formation or activate caspases (Riedl and Salvesen, 2007). Thus, the role of cytochrome *c* in the electron transport chain is even more basic than its role in apoptosis. Cytochrome *c* carries electrons from the mitochondrial inner membrane protein complex III to complex IV and is essential for the generation of the mitochondrial membrane potential ($\Delta\psi$) that drives ATP formation. Interaction between cytochrome *c* and both mitochondrial and cytosolic tRNA has been detected in healthy cells. It is thus possible that tRNA may regulate the electron transport chain and oxidative phosphorylation. It remains to be seen whether the cytochrome *c*:tRNA interaction provides a way to coordinate protein translation both inside and outside mitochondria with energy production. An additional point of interest is that mutations in mitochondrial tRNA are widely associated with human diseases (Wallace, 2005). It is generally assumed that these mutations affect mitochondrial protein synthesis, although protein synthesis defects have not always been detected in such cases. It would be interesting to test whether these tRNA mutations might affect cytochrome *c* action.

Cytochrome *c* and tRNA are both ancient and extensively studied molecules. The surprising interaction between them underscores how much we have yet to learn about other well-studied biologic systems. This interaction is a striking example of direct regulation of protein function by RNA that is very different from well-established models in which RNA acts principally at the level of gene expression. The protein and RNA worlds may be further intertwined than previously imagined.

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ABBREVIATIONS

aaRS, aminoacyl-tRNA synthetase; Apaf-1, the apoptotic protease activating factor-1; CARD, caspase recruitment domain; CAS, cellular apoptosis susceptibility protein; DISC, death inducing signaling complex; D-stem, dihydrouridine stem; GSH, glutathione; IAP, inhibitor of apoptosis; LysRS, lysyl-tRNA synthetase; MOMP, mitochondrial outer membrane permeabilization; PHAPI, putative HLA-DR-associated protein 1; pol III, RNA polymerase III; Pro-T, prothymosin-α; XIAP, X-linked IAP

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