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Molecular Basis and Consequences of the Cytochrome c-tRNA Interaction*

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The intrinsic apoptosis pathway occurs through the release of mitochondrial cytochrome c to the cytosol, where it promotes activation of the caspase family of proteases. The observation that tRNA binds to cytochrome c revealed a previously unexpected mode of apoptotic regulation. However, the molecular characteristics of this interaction, and its impact on each interaction partner, are not well understood. Using a novel fluorescence assay, we show here that cytochrome c binds to tRNA with an affinity comparable with other tRNA-protein binding interactions and with a molecular ratio of ~3:1. Cytochrome c recognizes the tertiary structural features of tRNA, particularly in the core region. This binding is independent of the charging state of tRNA but is regulated by the redox state of cytochrome c, with a molecular ratio of ~3:1. Cytochrome c recognizes the tertiary structural features of tRNA, particularly in the core region. This binding is independent of the charging state of tRNA but is regulated by the redox state of cytochrome c. Compared with reduced cytochrome c, oxidized cytochrome c binds to tRNA with a weaker affinity, which correlates with its stronger pro-apoptotic activity. tRNA binding both facilitates cytochrome c reduction and inhibits the peroxidase activity of cytochrome c, which is involved in its release from mitochondria. Together, these findings provide new insights into the cytochrome c-tRNA interaction and apoptotic regulation.

Cytochrome c is a heme-containing protein that normally resides in the mitochondrial inter-membrane space. It carries electrons from cytochrome c reductase (the cytochrome b-c1 complex) to cytochrome c oxidase as part of the electron transport chain that builds an electrochemical gradient driving the synthesis of ATP. This function of cytochrome c may be conserved over 1.5-billion years of eukaryotic evolution (1). In vertebrate cells, cytochrome c has taken on an additional role as a critical inducer of apoptosis or programmed cell death, which eliminates unwanted or harmful cells (1, 2). Apoptosis can occur through either of two major apoptotic pathways. The intrinsic apoptotic pathway is activated by intracellular stimuli such as DNA damage, oncogene activation, and developmental information. The extrinsic apoptotic pathway responds to extracellular stimuli via cell surface death receptors. The intrinsic pathway is evolutionarily more conserved than the extrinsic pathway, and it can be activated by the extrinsic pathway to amplify the apoptotic response. The defining event in the intrinsic pathway is the release of cytochrome c from mitochondria into the cytosol, where it binds to Apaf-1 (apoptotic protease activating factor-1) in the presence of ATP or dATP, facilitating the assembly of the oligomeric apoptosome (3–5). The apoptosome recruits and activates the initiator caspase, caspase-9 (6). Caspase-9 subsequently activates executioner caspases, leading to the cleavage of a large number of cellular proteins and eventually cellular death (7–9).

The activation of caspases by cytochrome c is intricately regulated. Release of cytochrome c is facilitated by the oxidation of cardiolipins, which anchor cytochrome c on the inner mitochondrial membrane, and by mitochondrial outer membrane permeabilization, a process that is regulated by members of the B-cell lymphoma protein-2 (Bcl2) family (1). In the cytoplasm, the ability of cytochrome c to activate caspases is modulated by its redox state, with the oxidized form showing a much more potent activity compared with the reduced form (10). Effective assembly of the apoptosome requires, in addition to cytochrome c and (d)ATP, the proteins HSPr70, cellular apoptosis susceptibility protein, and the PHAPI tumor suppressor (11, 12). Apoptosome formation is inhibited by the oncoprotein prothymosin-α (11). Nucleic acid, specifically transfer RNA (tRNA), is also implicated in the regulation of cytochrome c-mediated caspase activation (13).

tRNA is responsible for the interpretation of nucleic acid sequences as amino acid sequences during protein synthesis in all known forms of life (14, 15). Mature tRNAs are 73–93 ribonucleotides in length and fold into a cloverleaf secondary and L-shaped tertiary structure. tRNA is “charged” by conjugation with an amino acid at the conserved 3′-CCA sequence, which resides at one end of the L-shaped structure. Opposite this end, a three-nucleotide anticodon sequence pairs with a specific mRNA codon and enables the translation of the codon into a specific amino acid. tRNA interacts with a number of proteins and other RNAs during its maturation, transport, aminoacylation (“charging”), and movement in and out of the ribosome. It also has a high degree of functional versatility in addition to protein synthesis. Non-canonical functions of tRNA include...
priming reverse transcription of specific viral genomes (16) and stimulating gene expression in response to amino acid deprivation (17).

We previously showed that tRNA binds directly to cytochrome c. The cytochrome c-tRNA binding prevents cytochrome c from interacting with Apaf-1 and activating apoptosis (13). This finding indicates that cytochrome c, in addition to supporting ATP production and to promoting apoptosis, is a tRNA-binding protein. However, although all cytosolic and mitochondrial tRNAs appear to participate in the interaction, the molecular basis remains unknown. Also mysterious is the effect of tRNA association on the redox state and peroxidase activity of cytochrome c, which have been implicated in the release of cytochrome c and the activation of the caspase cascade. Here, we further characterize the dynamics of the interaction between tRNA and cytochrome c, the influence of novel factors, and its consequences for each interaction partner. This study elucidates the basic tenets of an ancient molecular interaction that has important consequences for apoptosis.

**Experimental Procedures**

Reagents—A 78-nucleotide DNA encoding the sequence of human initiator tRNA\textsubscript{Met} was synthesized by Integrated DNA Technologies (IDT; Coralville, IA). Cy3 was purchased from AAT Bioquest (Sunnyvale, CA), Cy5 from Lumiprobe (Hanover, Germany), and 2-aminopurine (2AP)\textsuperscript{4} triphosphate from TriLink Biotech (San Diego). The following reagents were purchased from Sigma: bovine heart and yeast cytochrome c; total tRNA from bakers’ yeast, ribosomal RNA, polyadenylic acid, NADH, NADPH, FAD, NaBH\textsubscript{4}, proflavine, ascorbic acid, and potassium ferricyanide. Onconase was provided by the Alfacell Corp. (Somerset, NJ).

Fluorophore-labeled tRNAs—A fluorescent tRNA based on the tRNA\textsubscript{Cy5} sequence in Escherichia coli was prepared by \textit{in vitro} reconstitution. E. coli tRNA\textsubscript{Cy5} (etRNA\textsubscript{Cy5}, Fig. 1A) has been well characterized (18, 19), and its crystal structure was determined (20). A 5’-fragment encoding nucleotides G1 to C16 was chemically synthesized by IDT with the Cy3 fluorophore (Fig. 1B) attached to the 5’-end (position 1) through a phosphodiester linkage. A 3’-fragment encoding G18 to A76 was synthesized by \textit{in vitro} transcription, using T7 RNA polymerase, and was gel-purified. The two fragments were joined by T4 RNA ligase I in a 3:1 molar ratio of the short versus long fragment with a 70% yield (Fig. 1C). The ligated full-length tRNA (Cy3-etRNA\textsubscript{Cy5}) was separated from individual fragments by a denaturing gel, heated at 85 °C, and re-annealed at 37 °C in the presence of Mg\textsuperscript{2+}. Similar procedures were used to prepare a human elongator tRNA\textsubscript{Met} (htRNA\textsubscript{Met}) and a human tRNA\textsubscript{Phe} (htRNA\textsubscript{Phe}) (Fig. 1A) that were labeled with Cy3 and Cy5, respectively, at the 5’-end (Cy3-htRNA\textsubscript{Cy5} and Cy5-htRNA\textsubscript{Phe}) (Fig. 1B). To generate the 2AP-labeled \textit{E. coli} tRNA\textsubscript{Val} (2AP-etRNA\textsubscript{Val}) (Fig. 1B), an \textit{E. coli} tRNA\textsubscript{Val} transcript that terminated at nucleotide position 75 was generated. 2AP was added to nucleotide position 76 using the CCA-adding enzyme from \textit{E. coli} and the triphosphate form of 2AP. The labeled tRNA was separated from unlabeled species on a denaturing PAGE, 7M urea gel. The full-length tRNA was excited from gels, recovered by ethanol precipitation, and resuspended in the TE buffer. The profilavine-labeled \textit{E. coli} tRNA\textsubscript{Cy5} (Prf-etRNA\textsubscript{Cy5}) was prepared by inserting profilavine (Fig. 1B) to the D-loop as described (Fig. 1D) (21, 22).

Binding Affinity of Cytochrome c with Fluorescent tRNAs—Each fluorescent tRNA was titrated with bovine or yeast cytochrome c from 0.1 to 24.4 \muM in the binding buffer (20 mM HEPES, pH 7.5, 35 mM KCl, 2.5 mM MgCl\textsubscript{2}, 0.5 mM EDTA, and 1 mM DTT) at room temperature, and the fluorescence emission was monitored. The peak intensity at 563 nm for Cy3 was corrected for the inner filter effect for each cytochrome c concentration, and the corrected data as a function of cytochrome c concentration were fit to a hyperbola equation to derive the \(K_d\) value.

Stoichiometry of Cytochrome c-tRNA Interaction—The stoichiometry of cytochrome c binding to tRNA was determined by monitoring the fluorescence quenching of Cy3-etRNA\textsubscript{Cy5} and Cy5-htRNA\textsubscript{Phe} on a Photon Technology International instrument model QM-4 as described (23). The binding was performed at room temperature in a buffer containing 20 \muM labeled tRNA, 200 \muM HEPES, pH 7.5, 50 mM NaCl, 5% sucrose, and 5 mM DTT. The bovine cytochrome c was titrated from 0 to 454 \muM, with the cytochrome c/tRNA molar ratio ranging from 0 to 22.7. The Cy3-etRNA\textsubscript{Cy5} was excited at 550 nm, and the emission was monitored from 558 to 650 nm at room temperature. The Cy5-htRNA\textsubscript{Phe} was excited at 640 nm, and the emission was monitored from 655 to 720 nm. The emission peaks at 565 nm for Cy3-tRNA and at 662 nm for Cy5-tRNA were recorded and corrected for the inner filter effect, according to the formula, \(F_{corr} = F_{obs} \times antilog((A_{excitation} + A_{emission})/2)\), where \(F_{corr}\) is the corrected fluorescent signal at the peak wavelength, and \(F_{obs}\) is the observed fluorescent signal at the peak wavelength.

Surface Plasmon Resonance (SPR) Assay—The association of various nucleic acids with cytochrome c was assessed by surface plasmon resonance using a BIAcore 3000 system (GE Healthcare). Cytochrome c from bovine heart was immobilized on a CM5 sensor chip (BIAcore) by amine coupling. Each nucleic acid was individually injected onto the immobilized cytochrome c. Binding interaction was performed in a low salt buffer (20 mM HEPES, 20 mM KCl, 2.5 mM MgCl\textsubscript{2}, 0.5 mM EDTA) or in a buffer with physiologic ionic strength (by adding KCl to 135 mM and NaCl to 10 mM to the low salt buffer). After each binding experiment, the chips were washed with a 0.5 M NaCl regeneration solution.

**CCA Addition and tRNA Charging Reactions**—The transcript of \textit{E. coli} tRNA\textsubscript{Val} was synthesized up to C75 and was internally labeled in the transcription reaction containing a standard reaction mixture (24). The putative CCA was then added, and tRNA\textsubscript{Val} was purified by gel electrophoresis (7M urea) and desalted by dialysis. An aliquot (0.01 \muM) of the transcript was used in each reaction, and the reactions were incubated at room temperature for 30 min. The reactions were terminated by adding a 5X stop buffer (400 mM CH\textsubscript{3}COOH, 300 mM NaCl, 100 mM DTT, 100 mM EDTA), and the samples were heated at 75 °C for 5 min. After denaturation, the reactions were cooled on ice and subsequently loaded onto a standard 15% polyacrylamide gel (5M urea) and electrophoresed for 12 h at 150 V. However, although all cytosolic and mitochondrial tRNAs appear to participate in the interaction, the molecular basis remains unknown. Also mysterious is the effect of tRNA association on the redox state and peroxidase activity of cytochrome c, which have been implicated in the release of cytochrome c and the activation of the caspase cascade. Here, we further characterize the dynamics of the interaction between tRNA and cytochrome c, the influence of novel factors, and its consequences for each interaction partner. This study elucidates the basic tenets of an ancient molecular interaction that has important consequences for apoptosis.
trace amount of \( [\alpha^{32}\text{P}] \text{ATP} \). The addition of A76 to the gel-purified labeled tRNA\(^{\text{Val}} \) (0.1 \( \mu \text{M} \)) was catalyzed by human CCA adding enzyme (2 \( \mu \text{M} \)), in the presence or absence of oxidized bovine cytochrome c (15 \( \mu \text{M} \)). The assay was performed at 37 °C for 0–1 s on a KinTek RQF-3 instrument in the reaction buffer previously described (24–26).

tRNA charging assay was performed using etRNA\(^{\text{Cys}} \) as the substrate and \( E. \ coli \) cysteinyl-tRNA synthetase (CysRS) as the enzyme, as described previously (27–29). The efficiency of charging was monitored using \([\text{35S}]\text{cysteine} \). After the reaction, tRNA was acid-precipitated on filter pads, and the radioactivity of \([\text{35S}]\text{cysteine} \) attached to each tRNA was quantified by scintillation counting (30).

**Cytochrome c Peroxidase Activity**—The peroxidase activity was determined by incubation of cytochrome c with enhanced chemiluminescence solution and measuring light emission using an illuminometer.

**Cytochrome c Oxidation and Reduction**—To generate the reduced protein, bulk cytochrome c was incubated with excess ascorbic acid and was then purified using a Sepharose column (GE Healthcare). To generate the oxidized protein, bulk cytochrome c was incubated with potassium ferricyanide and purified similarly. The oxidation and reduction were confirmed by measuring absorbance at 550 and 560 nm as well as by colorimetric inspection of the purified protein. In cytochrome c oxidation and reduction assays, the redox state was monitored by continuous measurement of absorption at 550 nm.

**Results**

**Fluorescence-based Assay for the Cytochrome c-tRNA Interaction**—We previously analyzed the cytochrome c-tRNA interaction using electrophoretic mobility shift assays (13). To provide an independent and quantitative evaluation of this interaction, we developed a fluorescence-based assay. Cytochrome c does not appear to strongly discriminate among various cytosolic and mitochondrial tRNA species (13), suggesting that prokaryotic tRNA may be just as capable of interacting with cytochrome c as eukaryotic tRNA. We used \( E. \ coli \) tRNACys (etRNACys) and tRNAVal (etRNAVal) and human elongator tRNAMet (htRNAMet) and tRNAPhe (htRNAPhe) as models (Fig. 1A). These tRNAs were labeled at the 5'-end with either Cy3 (for etRNACys, htRNAMet, and htRNAPhe), or the 3'-end labeled with 2AP (for etRNAVal). B, schematic representation of tRNAs labeled with Cy3 (maximal emission wavelength or \( \lambda_{\text{em}} = 570 \text{ nm} \) ) or Cy5 (\( \lambda_{\text{em}} = 670 \text{ nm} \) ) at the 5'-end, 2-AP (\( \lambda_{\text{em}} = 320 \text{ nm} \) ) at the 3'-end, and Prf (\( \lambda_{\text{em}} = 515 \text{ nm} \) ) in the D-loop. C, construction of tRNA labeled with Cy3 at the 5'-end. The 5'-Cy3 fragment and the 3'-tRNA transcript fragment were joined together using T4 RNA ligase in the presence of ATP. D, generation of tRNA labeled with proflavine attached to a ureidopropional group at the D-loop.

**FIGURE 1.** Generation of fluorescence-labeled tRNAs. A, sequence and cloverleaf structure of tRNAs used in this study. Species are \( E. \ coli \) tRNACys (etRNACys) and tRNAVal (etRNAVal) and human elongator tRNAMet (htRNAMet) and tRNAPhe (htRNAPhe). Arrows indicate the positions of joining between a synthetic Cy3- or Cy5-labeled 5'-fragment and an \textit{in vitro}-transcribed 3'-fragment (for etRNACys, htRNAMet, and htRNAPhe), or the 3'-position labeled with 2AP (for etRNAVal). B, schematic representation of tRNAs labeled with Cy3 (maximal emission wavelength or \( \lambda_{\text{em}} = 570 \text{ nm} \) ) or Cy5 (\( \lambda_{\text{em}} = 670 \text{ nm} \) ) at the 5'-end, 2-AP (\( \lambda_{\text{em}} = 320 \text{ nm} \) ) at the 3'-end, and Prf (\( \lambda_{\text{em}} = 515 \text{ nm} \) ) in the D-loop. C, construction of tRNA labeled with Cy3 at the 5'-end. The 5'-Cy3 fragment and the 3'-tRNA transcript fragment were joined together using T4 RNA ligase in the presence of ATP. D, generation of tRNA labeled with proflavine attached to a ureidopropional group at the D-loop.
To assess whether the attachment of the fluorophores at the 5′-end affects the function of tRNA, we used etRNACys as an example and compared charging to the 3′-end of unlabeled and the 5′-end Cy3-labeled etRNACys. Using [35S]cysteine as the substrate and *E. coli* CysRS as the enzyme, we analyzed the attachment of [35S]cysteine to each tRNA over time. As shown in Fig. 2A, Cy3-etRNACys was charged to 85% of the unlabeled etRNACys. This indicates that the labeling at the 5′-end does not interfere with charging.

Interaction of these tRNAs with cytochrome *c* was measured by fluorescence quenching of the labeled tRNAs following cytochrome *c* addition (Fig. 2B). Quenching data were fit to a hyperbolic equation to obtain the *K_d* value of the interaction, assuming a two-state (bound or unbound) model following correction for the inner filter effect and nonspecific binding.

Cytochrome *c* Binds to tRNA with an Affinity Comparable with Other tRNA-binding Proteins and Recognizes the Tertiary Structure of tRNA—We determined the affinity of bovine heart cytochrome *c* to Cy3-etRNA<sub>Cys</sub> and observed a *K_d* in the range of 1–3.5 μM (Fig. 2, C and D, and Table 1). Importantly, this affinity was comparable with those of known tRNA-binding proteins, including aminoacyl-tRNA synthetase (aaRS) (1–3 μM) (27, 31, 32) and CCA-adding enzyme (tRNA-nucleotidyltransferase) (0.8–3.3 μM) (25, 26). To corroborate this finding, we also analyzed cytochrome *c* binding to Cy3-htRNAMet and 2AP-etRNA<sub>Val</sub>. Cytochrome *c* bound to these two tRNAs with affinities similar to Cy3-etRNA<sub>Cys</sub> (*K_d* of 4.8 ± 0.7 and 5.1 ± 0.9 μM, respectively; Table 1). Therefore, analysis of three unrelated tRNA species labeled with distinct fluorophores at different ends showed that cytochrome *c* bound to tRNA species with an affinity akin to those of other tRNA-binding proteins.

Compared with Cy3-etRNA<sub>Cys</sub>, cytochrome *c* bound to the D-loop-labeled Prf-etRNA<sub>Cys</sub> with a substantially reduced affinity (*K_d* of 9.6 ± 2.8 μM, Table 1). This might be due to proflavine in the D-loop interfering with cytochrome *c* binding directly or with folding of the tertiary structure of tRNA, both scenarios suggesting that the tRNA tertiary structure is required for high affinity interaction with cytochrome *c*. We also used a Cy3-labeled fragment of etRNA<sub>Cys</sub> encoding nucleotides 1–16 (Cy3-oligonucleotide), which resembled a microRNA or a tRNA-derived fragment and lacked the tertiary
**Molecular Interaction of Cytochrome c and tRNA**

**TABLE 1**

<table>
<thead>
<tr>
<th>RNA</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3-etRNA$^{Cys}$</td>
<td>1−3.5</td>
</tr>
<tr>
<td>Cy3-htRNA$^{Met}$</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>2AP-etRNA$^{Cys}$</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>Prf-etRNA$^{Cys}$</td>
<td>9.6 ± 2.8</td>
</tr>
<tr>
<td>Cy3-oligonucleotide</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Dissociation constant of bovine heart cytochrome c to different RNA

Measurement of the $K_d$ (tRNA) for cytochrome c by fluorescence titration is shown. *E. coli* tRNA$^{Cys}$ and human tRNA$^{Met}$ labeled with Cy3 at the 5’-end, *E. coli* tRNA$^{Cys}$ labeled with 2-AP on the 3’-end, or *E. coli* tRNA$^{Cys}$ labeled with Prf in the D-loop were used in the binding assay. The Cy3-oligonucleotide refers to the oligonucleotides corresponding to the sequence from positions 1 to 16 in *E. coli* tRNA$^{Cys}$, carrying Cy3 at the 5’-end. Data shown are mean ± S.D.

core structure (33). This fragment had greatly reduced affinity for cytochrome c ($K_d > 30$ μM, Table 1).

To extend these analyses, we generated a mutant of etRNA$^{Cys}$, in which three nucleotides in the D loop (U20, U21, and A22) were deleted, and the G48 nucleotide in the V-loop was changed to C (Fig. 2D, left). We have previously shown that the nucleotides in the D-loop and G48, which forms base pair with G15, are critical for the integrity of the core (18, 19, 34, 35). Thus, the resultant etRNA$^{Cys}$ mutant likely has an incomplete tertiary structure. Indeed, this mutant bound to cytochrome c with a $K_d$ of 5.5 ± 1.7 μM, which was a 5-fold increase relative to the $K_d$ of 1.0 ± 0.3 μM of the wild-type tRNA determined in the same experiment (Fig. 2D). Together, these results support the notion that cytochrome c recognizes the tertiary structural features of tRNA, particularly in the core region.

Surface Plasmon Resonance Analysis—To further independently assess the cytochrome c-tRNA interaction, we performed a surface plasmon resonance analysis using a BiaCore 3000 system. Bovine heart cytochrome c was immobilized on a CM5 sensor chip by amine coupling and tested for binding with total tRNA, ribosomal RNA (rRNA), a mixture of poly(A) RNAs, and a 78-nucleotide DNA oligonucleotide corresponding to a human initiator tRNA$^{Met}$. In a low salt buffer, only tRNA had distinct association and dissociation phases (Fig. 3, A–C). In a buffer with physiologic ionic strength, only tRNA binding was detectable above the baseline (Fig. 3D). These results again indicate that cytochrome c is a specific tRNA-binding protein.

Cytochrome c Associates with tRNA with a Stoichiometric Ratio of Three Cytochrome c Molecules for Each tRNA Molecule—We next interrogated the binding stoichiometry between tRNA and cytochrome c. We titrated a fixed amount of Cy3-etRNA$^{Cys}$ with increasing amounts of cytochrome c to shift the binding equilibrium in the direction of the bound complex. The titration produced a biphasic quenching of the Cy3-etRNA$^{Cys}$ fluorescence, with an initial steep phase followed by a second and much flatter phase (Fig. 3E). The two phases intersected at a molar ratio of cytochrome c to tRNA at ~3.0, indicating that three cytochrome c molecules bind to one tRNA molecule. Further fluorescence quenching was also observed at higher protein stoichiometries, likely indicating nonspecific formation of higher order complexes.

To confirm this binding stoichiometry, we tested Cy5-htRNA$^{Phe}$. Despite the use of a different fluorophore and a different tRNA, the titration maintained the same two phases that intersected at the cytochrome c/tRNA molar ratio of ~3.0 (Fig. 3F). The stoichiometry observed in these experiments is in agreement with the result of a recent study (36), and it further underscores the specificity of the cytochrome c-tRNA interaction.

Influence of the CCA-end of tRNA on Cytochrome c Binding—We next investigated whether some of the key features of tRNA and cytochrome c regulate their interaction. The addition of the CCA sequence is an essential step in the maturation of tRNA (14, 15). To determine whether the CCA sequence is required for cytochrome c binding, we used tRNA species with and without this sequence. An analysis of the interaction between Cy3-htRNA$^{Met}$ and either bovine or yeast cytochrome c showed no major difference regardless of the presence or absence of the CCA sequence (Table 2). Thus, the CCA sequence does not appear to affect cytochrome c binding.

Conversely, we assessed whether cytochrome c influences the CCA addition reaction. Using the human CCA-adding enzyme and under single-turnover conditions, we monitored the A76 addition to the transcript of *E. coli* tRNA$^{Val}$ that terminated with C75. Analysis of the time course of the reaction showed that the rate constant of the addition in the presence and absence of cytochrome c was virtually identical (14 versus 15 s$^{-1}$) (Fig. 4A). Thus, the action of the CCA-adding enzyme is not perturbed by cytochrome c.

We also tested whether CCA-adding enzyme and cytochrome c compete for binding to tRNA. We pre-assembled an etRNA$^{Cys}$-CCA-adding enzyme complex and performed the binding assay with a range of cytochrome c concentrations. The etRNA in the etRNA$^{Cys}$-CCA-adding enzyme complex was able to bind cytochrome c with a $K_d$ of 0.9 ± 0.3 μM, essentially the same as the binding of free tRNA with cytochrome c ($K_d$ of 1.0 ± 0.3 μM), determined under the same condition (Fig. 4B). In the crystal structure of a tRNA-CCA-adding enzyme complex, the CCA-adding enzyme binds to the top half of the tRNA L-shaped tertiary structure near the 3’-end (Fig. 4C) (37). Thus, our data indicate that the CCA-adding enzyme does not block the access of cytochrome c to the tRNA structure.

Influence of the Charging State of tRNA on Cytochrome c Binding—tRNA charging by cognate aaRS is fundamental to the translation of mRNA into protein. The state of tRNA charging is also an indicator of the nutritional state, and amino acid deprivation leads to rapid accumulation of uncharged tRNAs (14, 15). The experiments described above were performed with uncharged tRNA. To assess the influence of the charging state of tRNA, we compared uncharged and charged Cy3-etRNA$^{Cys}$ for binding to cytochrome c. Bovine cytochrome c bound to these two forms of tRNA with similar affinities ($K_d$ of 3.6 ± 0.8 and 3.3 ± 0.7 μM, respectively) (Fig. 4D). Thus, cytochrome c recognizes tRNA independent of its charging state.

To evaluate whether cytochrome c competes with aaRS for binding to tRNA, we compared the binding to cytochrome c of free etRNA$^{Cys}$ and etRNA$^{Cys}$ associated with CysRS. The etRNA molecules in these two states bound to cytochrome c with virtually the same affinities ($K_d$ of 1.0 ± 0.3 and 1.2 ± 0.3 μM, respectively) (Fig. 4E). In the crystal structure of the...
etRNA-Cys-CysRS complex, the CysRS enzyme binds to the inside of the tRNA L-shape (Fig. 4C) (38). The lack of competition between CysRS and cytochrome c is consistent with a model in which cytochrome c binds to the outside corner of the tRNA L-shape and hence is not in conflict with the binding by CysRS (Fig. 4C). It also suggests that labeling of tRNA at the 5′- or 3′-end unlikely interferes with its interaction with cytochrome c.

**Binding of Oxidized and Reduced Cytochrome c to tRNA**—Cytochrome c exists in either a reduced or an oxidized form, based on the oxidation state of the iron atom contained within the heme group. The oxidized form is much more potent than the reduced form in the activation of caspases (10). All experiments reported thus far employed oxidized cytochrome c. To test the influence of the redox state of cytochrome c, we compared binding of oxidized and reduced cytochrome c to tRNA directly. Oxidized bovine cytochrome c bound to Cy3-htRNAMet with a 2-fold weaker affinity compared with the reduced form (Kd = 4.8 ± 0.7 μM versus 2.5 ± 1.4 μM) (Fig. 5A, left). Interestingly, removal of the CCA sequence exacerbated the difference in binding between the two states (4-fold; Kd = 3.7 ± 0.4 versus 0.9 ± 0.2 μM) (Fig. 5A, right). We also tested cytochrome c from yeast, which, unlike its vertebrate counterpart, cannot induce caspase activation (39). Oxidized yeast cytochrome c displayed a weaker affinity to Cy3-htRNA^{Met} with a 2-fold weaker affinity compared with the reduced form (Kd = 4.8 ± 0.9 versus 3.4 ± 0.4 μM in the presence of CCA and Kd = 7.0 ± 2.0 versus 3.7 ± 0.7 μM in the absence of CCA) (Fig. 5B). The weaker binding of the oxidized form of mammalian cytochrome c to tRNA correlates with its stronger ability to activate apoptosis.

### TABLE 2

<table>
<thead>
<tr>
<th>tRNA</th>
<th>CCA end</th>
<th>Label</th>
<th>Bovine cyt c</th>
<th>Yeast cyt c</th>
</tr>
</thead>
<tbody>
<tr>
<td>htRNA^{Met}</td>
<td>CCA+</td>
<td>5'-Cy3</td>
<td>4.8 ± 0.7</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>htRNA^{Met}</td>
<td>CCA−</td>
<td>5'-Cy3</td>
<td>3.7 ± 0.4</td>
<td>7.0 ± 2.0</td>
</tr>
<tr>
<td>htRNA^{Met}</td>
<td>CCA−</td>
<td>5'-Cy3</td>
<td>3.7 ± 0.4</td>
<td>7.0 ± 2.0</td>
</tr>
<tr>
<td>htRNA^{Met}</td>
<td>CCA−</td>
<td>5'-Cy3</td>
<td>3.7 ± 0.4</td>
<td>7.0 ± 2.0</td>
</tr>
</tbody>
</table>

The data shown are mean ± S.D.
Effect of tRNA Binding on the Activity of Cytochrome c—Cytochrome c possesses peroxidase activity, which promotes the oxidization of cardiolipins early in apoptosis, facilitating the detachment of cytochrome c from cardiolipins and its subsequent release into the cytosol (40). To further investigate how the cytochrome c-tRNA interaction may affect cytochrome c function, we tested whether tRNA binding modulates the peroxidase activity of cytochrome c. In a luminescence assay, tRNA inhibited the peroxidase activity of cytochrome c in a dose-dependent manner (Fig. 6A). This finding raises the possibility that tRNA, if present in the inter-membrane space upon mitochondrial outer membrane permeabilization, might impede the oxidation of cardiolipins and regulate apoptosis at the level of cytochrome c release.

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tRNA Binding Facilitates Cytochrome c Reduction—RNA is extensively oxidized during apoptosis (41). To test whether cytochrome c is capable of directly oxidizing tRNA, we incubated cytochrome c with tRNA and observed the changes in absorbance at 550 nm, which monitors the reduced state. In the absence of tRNA, oxidized cytochrome c was stable for days. However, upon incubation with tRNA, the oxidized state was gradually converted to the reduced state, as indicated by the increase in the absorption at 550 nm (Fig. 6B). This increase was dependent on the concentration of cytochrome c. Importantly, pre-digestion of tRNA by addition of the nuclease Oncorase (ranpirnase) prevented cytochrome c reduction (Fig. 6C). These results suggest that tRNA, but not free NMPs, may serve as a substrate for oxidation by cytochrome c.
Discussion

Discovered by Keilin and co-workers (42, 43) in the 1920s as one of the color proteins involved in the respiratory chain, cytochrome c has an essential and evolutionarily conserved role in supporting aerobic eukaryotic life. More than 70 years later, a completely unanticipated role of cytochrome c in vertebrate cell death came to light, when Wang and co-workers (2) investigated the mechanism of caspase activation. The dichotomy of the dual roles of cytochrome c mechanistically links cell life and death and is fundamental to the evolutionary covenant required for multicellular life. Thus, virtually all vertebrate cells, by depending on cytochrome c for survival, carry this suicide pill for use when and where needed.

tRNA is even more evolutionarily ancient and fundamental to life (14, 15). Its function as the adaptor molecule in protein synthesis is based on the L-shaped tertiary structure that

FIGURE 6. tRNA inhibits the peroxidase activity of cytochrome c and promotes the reduction of cytochrome c. A, tRNA inhibits cytochrome(cyt) c-peroxidase activity. Left, luminescence emission in an enhanced chemiluminescence assay, representing peroxidase activity, was linear with the amount of cytochrome c. Right, cytochrome c peroxidase activity, monitored by luminescence using Lumigen TMA-6 and hydrogen peroxide, in the presence or absence of tRNA. Concentrations of cytochrome c and tRNA (in μM) are shown. B, tRNA reduces oxidized cytochrome c. Left, oxidation of cytochrome c by peroxide as monitored by A_{550 nm}. Right, incubation of tRNA in a buffer with physiologic ionic strength (150 mM) with oxidized cytochrome c at molar ratios of 1:2, 1:1, or 2:1 led to increases in A_{550 nm} indicating the reduction of cytochrome c over time. C, tRNA must be intact to reduce oxidized cytochrome c. tRNA-dependent reduction of cytochrome c is inhibited upon the degradation of tRNA by Onconase (ranpirnase). Error bars indicate S.D.
Molecular Interaction of Cytochrome c and tRNA

simultaneously recognizes a genetic codon and an amino acid. This tertiary structure allows for tRNA to interact with both general enzymes, such as the CCA-adding enzyme for 3'-end maturation, and specific enzymes, such as aaRS for charging. The L-shaped tertiary structure also affords tRNA non-conventional roles in cells, including priming reverse transcription (16) and, for uncharged tRNA, sensing nutrient deprivation (17).

The identification of the association between cytochrome c and tRNA revealed a previously unrecognized connection between two fundamental molecules in life and signified novel biochemical properties of each (13). The findings presented here show that cytochrome c binds to tRNA with an affinity comparable with other tRNA-binding proteins (Fig. 2 and Table 1). Interestingly, cytochrome c binds tRNA without sequence specificity, a property unlike aaRS and more similar to CCA-adding enzyme. Cytochrome c likely recognizes features of the tertiary structure of tRNA, particularly in the core region. The binding stoichiometry of three cytochrome c molecules to a single tRNA molecule (Fig. 3) additionally suggests that multiple cytochrome c molecules are coordinated to recognize a single tRNA. Interestingly, cytochrome c does not compete with either aaRS or CCA-adding enzyme. This could be accounted for in a model in which three cytochrome c molecules bind to the outside corner of the tRNA L-shape in a way that does not interfere with the binding of aaRS to the inside of the L-structure or with the binding of CCA-adding enzyme to the outside of the L-structure near the 3’-end (Fig. 4C).

In cells, tRNAs are extensively modified. These modifications can modulate the structure, function, and stability of tRNAs (15). The tRNAs used in this study lack the post-transcriptional modifications. Nevertheless, our data suggest that cytochrome c binds to the outside corner of the tRNA tertiary core, which is not extensively modified relative to the anticodon loop region. Post-transcriptional modifications to the outside corner of the tRNA tertiary core primarily consist of dihydrouridine residues, which have also been used extensively to introduce fluorophores to monitor tRNA dynamics on the ribosome (21, 22). This indicates that these residues themselves are not critical for the intra-molecular folding of the tRNA tertiary core. Therefore, we suggest that the use of unmodified tRNAs does not affect the binding with cytochrome c.

Apoptosis is tightly regulated at many levels (44, 45). The inhibition of the cytochrome c-Apaf-1 binding by tRNA may present an important cytosolic regulatory mechanism. Thus, tRNAs can bind to cytochrome c that is released into the cytoplasm, providing an inhibitory mechanism for apoptosis and linking cellular sensitivity to apoptotic stimuli with the state of protein synthesis (46–48). This study provides additional insights into the mechanism by which the apoptotic activity of cytochrome c is regulated. Oxidation of cytochrome c stimulates its apoptotic activity, whereas reduction of cytochrome c inhibits it (10). In apoptotic cells, cytochrome c released into the cytosol is likely maintained in the oxidized form by mitochondrial cytochrome c oxidase, which can act on the released cytochrome c due to the permeability of the mitochondrial outer membrane (10). By contrast, in cases where the release of cytochrome c fails to induce apoptosis, cytochrome c may be held in the reduced form by reduced glutathione (49). Our observation that tRNA binds to oxidized cytochrome c with a weaker affinity than to reduced cytochrome c (Fig. 5) provides a possible explanation for the different apoptotic activity of these two forms of cytochrome c.

tRNA can also convert cytochrome c from the oxidized to the reduced form (Fig. 5). The most likely explanation is that tRNA ribonucleotides contact with the heme group of cytochrome c and donate an electron to the ferric ion (Fe3+). This likely contributes to the reduction of the apoptotic activity of cytochrome c. The same redox reaction can also blunt the measured peroxidase activity of cytochrome c (Fig. 6). Because the peroxidase activity of cytochrome c is involved in the oxidation of cardiolipins and subsequent release of cytochrome c from the cristae space (the space created by the invaginations of the inner membrane) to the cytosol (40), tRNA might also inhibit this function of cytochrome c if it has access to cardiolipid-bound cytochrome c. This scenario seems possible because of the permeability of mitochondrial outer membrane and the remodeling of the cristae space during apoptosis. Thus, the redox reaction between cytochrome c and tRNA suggests a broad function by which tRNA impairs the pro-apoptotic activity of cytochrome c, beyond the disruption of the cytochrome c-Apaf-1 interaction. In contrast, cytochrome c-mediated oxidation could cause damage to tRNA and thus may impair translation, further promoting cell death.

In addition to representing new modes of regulation and function of cytochrome c in apoptosis, the discovery of the cytochrome c-tRNA interaction revealed a previously completely unanticipated role of tRNA. Although it had been appreciated that the three-dimensional structure of tRNA endows it with functions beyond gene expression, a direct function in cell fate decision is especially notable. The inhibitory role of tRNA in apoptosis may raise the threshold of apoptosis in cells that are highly active in protein synthesis, a sensible mechanism given the likely utility of these cells to the organism.

A recent study showed that tRNA halves, which are generated by endonucleolytic cleavage in the anticodon loop in response to oxidative and other stresses (50), bind to cytochrome c and confer resistance to apoptosis (51). We have shown that a pair of tRNA halves, separated by a nick in the anticodon loop, can nonetheless retain the L-shaped structure (26), which may account for their ability to bind to tRNA. Such tRNA halves represent an intriguing example whereby tRNA-mediated apoptotic inhibition is regulated physiologically. This mechanism may also be usurped under pathological conditions, including cancer. Mammalian cytoplasmic tRNAs are transcribed by RNA polymerase III, which is inhibited by the tumor suppressors p53 and the retinoblastoma protein (Rb) and activated by the oncoproteins, including c-Myc and Ras (52). Mutations in these tumor suppressors/oncogenes cause tRNA levels to rise in tumor cells, and high tRNA levels are required for proliferation and tumorigenesis (52). A better understanding of the molecular interaction between cytochrome c and tRNA and the regulation of this interaction should reveal evolutionarily conserved mechanisms that govern apoptosis, metabo-
Molecular Interaction of Cytochrome c and tRNA

domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability. Proc. Natl. Acad. Sci. U.S.A. 86, 4579–4583
Molecular Interaction of Cytochrome c and tRNA
