Ribosomes Specifically Bind to Mammalian Mitochondria via Protease-sensitive Proteins on the Outer Membrane*

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The interaction of ribosomes with specific components of membranes is one of the central themes to the co-translational targeting and import of proteins. To examine ribosome binding to mammalian mitochondria, we used ribosome-nascent chain complexes (RNCs) to follow the in vitro binding of ribosomes that correspond to the initial targeting stage of proteins. Mitochondria were found to contain a limited number of RNC binding sites on the outer membrane. It required more than twice the amount of non-translating ribosomes to inhibit RNC binding by one-half, indicating that RNCs have a competitive binding advantage. In addition, we found that RNCs bind mainly through the ribosomal component and not the nascent chain. RNCs bind via protease-sensitive proteins on the outer membrane, as well as by protease-insensitive components suggesting that two classes of receptors exist. We also show that binding is sensitive to cation conditions. Nearly all of the binding was inhibited in 0.5 M KCl indicating that they interact with the membrane primarily through electrostatic interactions. In addition, disruption of RNC structure by removing magnesium causes the complete inhibition of binding under normal binding conditions indicating that it is the intact ribosome that is crucial for binding and not the nascent chain. These findings support the hypothesis that the outer mitochondrial membrane contains receptors specific for ribosomes, which would support the conditions necessary for co-translational import.

Newly synthesized proteins are targeted to, and translocated across, membranes either post-translationally or co-translationally. Most mitochondrial proteins are encoded in the nucleus, synthesized by free cytosolic ribosomes, and translocated into mitochondria post-translationally (1); however, evidence suggests that some proteins may be imported co-translationally (2). The co-translational model of protein import into mitochondria was first put forth after in vitro studies in yeast found that cytosolic polyosomes: 1) are associated with mitochondria; 2) the number bound is dependent on the metabolic state of the cells; 3) are translationally active; and 4) contain mRNA encoding mitochondrial proteins (3–8). In addition, fully synthesized, unprocessed proteins are not found under normal growth conditions in yeast and treatment with cycloheximide to inhibit translation disrupts mitochondrial protein import in vitro and in vivo, suggesting that proteins are translated into mitochondria either rapidly after synthesis or co-translationally (9–11). The translation and import of some mitochondrial proteins have also been found to be tightly coupled in mammalian cells (12, 13).

Direct interactions between ribosomes and mitochondria are central to the co-translational hypothesis and recent work in our laboratory and others has focused on the role that ribosomes, their associated nascent proteins, and cytosolic chaperones play in the import of mitochondrial proteins. For example, mutations in the nascent polypeptide-associated complex (NAC), which interacts with both the ribosome and the emerging nascent chain, lead to defects in protein targeting to both mitochondria and endoplasmic reticulum (ER) in yeast (14). NAC has also been shown in vitro to be directly involved in the co-translational import of the mitochondrial matrix protein malate dehydrogenase (mMDH), where it has been hypothesized that NAC interacts with the nascent precursor protein and assists in the docking of ribosomes to the mitochondrial surface (15, 16). Yeast mutants lacking NAC do not have defects in translation, but do have a significant decrease in the number of ribosomes bound to mitochondria and although the levels of most mitochondrial proteins are unchanged, some of the proteins that are thought to be imported more efficiently co-translationally are decreased (17). This supports the hypothesis that there is a subclass of mitochondrial proteins that may be most efficiently imported co-translationally (18).

We previously reported that ribosomes specifically bind mammalian mitochondria through a process that is controlled by a GTP-dependent step, which is similar to co-translational import in the ER (19). We found that ribosome binding to mitochondria was not stable at physiological levels of GTP, whereas ATP and GDP only mildly affected binding. In addition, we found that a nascent chain containing a proper mitochondrial targeting sequence behaved like unprocessed ribosomes in the presence of GTP, whereas nascent chains not containing a mitochondrial targeting sequence behaved like unprogrammed ribosomes. Also, the endogenous ability of mitochondria to hydrolyze GTP was greatly accelerated when GTPase-depleted ribosomes were added to mitochondria, whereas ribosome-mitochondria interactions had no effect on ATP hydrolysis. These findings indicated that ribosomes bind tightly to mitochondria through a process that involves a GTP-depend-
ent step and the nascent chain bound to the ribosome. This is similar to what occurs at the ER membrane where GTP and the signal sequence are involved in ribosomes interacting with both signal recognition particle and the translocation apparatus at the ER membrane (20).

Ribosomes specifically interact with the proteins that make up the translocation pore at target membranes during co-translational translocation and this interaction has been conserved (21). We have developed binding assays using ribosome-nascent chain complexes to determine the conditions under which ribosomes bind mammalian mitochondria. We demonstrate that ribosomes bind mitochondria through electrostatic interactions with proteins on the outer membrane that are susceptible to proteolysis. We also present evidence that the ribosomal component of the ribosome-nascent chain complex is central to binding. These findings support the hypothesis that the outer mitochondrial membrane contains receptors that are specific for ribosomes.

EXPERIMENTAL PROCEDURES

Preparation of High Salt-washed Mitochondria—Crude mitochondria were isolated from Sprague-Dawley rat livers as previously described (19). Mitochondria were washed twice with mitochondria isolation buffer (MIB, 10 mM Tris, pH 7.4, 70 mM mannitol, and 220 mM sucrose) to remove excess EDTA and high salt. Reduction of ER contamination was confirmed by assessing the activity of arylesterase and by electron microscopy of mitochondria preparations using previously described methods (19, 23, 25) (data not shown). Protein concentration was determined using the BCA protein assay kit (Promege, Madison, WI), brought to 10 mg/ml in MIB, and used immediately. The intactness of all preparations was assessed by determining the respiratory control ratio using a Clark electrode and was between 5 and 6 (data not shown). Freshly isolated, high salt-washed mitochondria were used for all experiments.

Construction of the "Mitochondrial Malate Dehydrogenase-Methionine 6" Plasmid—To add 6 methionines to the COOH-terminal of the mMDH nascent chain, the "methionine insert" plasmid was first created, which allows for a DNA construct with EcoRI and KpnI restriction sites to be inserted so that 6 methionines are added to the COOH-terminal of the nascent chain. A 45 bp double-stranded DNA construct was inserted by using previously described methods to reduce ER contamination and to strip the mitochondria of endogenously bound ribosomes (19, 23, 24). Mitochondria were washed twice with mitochondria isolation buffer (MIB, 10 mM Tris, pH 7.4, 70 mM mannitol, and 220 mM sucrose) to remove excess EDTA and high salt. Reduction of ER contamination was confirmed by assessing the activity of arylesterase and by electron microscopy of mitochondria preparations using previously described methods (19, 23, 25) (data not shown). Protein concentration was determined using the BCA protein assay kit (Promege, Madison, WI), brought to 10 mg/ml in MIB, and used immediately. The intactness of all preparations was assessed by determining the respiratory control ratio using a Clark electrode and was between 5 and 6 (data not shown). Freshly isolated, high salt-washed mitochondria were used for all experiments.

Preparation of Ribosome-Nascent Chain Complexes—Truncated mMDH-Met6 mRNA lacking a stop codon was synthesized by in vitro transcription of the mMDH-Met6 insert plasmid linearized with XbaI. Cell-free translations were performed in a reticulocyte lysate system, as previously described, to produce ribosome-nascent chain complexes (RNCs) carrying polypeptides that were 93 amino acids in length (ribo- mMDH-Met6) (20). Translations contained nucleoside-triphosphate, reticulocyte lysate (70%, v/v) (Promega), 20 μM minus methionine amino acid mixture, 0.9 mM Tris Redivue L-[35S]methionine (Amersham Biosciences), the in vitro transcription reaction (12.5%, v/v), and 0.3 units/ml RNasin. The translation reaction was inhibited with 1 mM cycloheximide, brought up in 8 volumes of isolation buffer to a final concentration of 100 mM KOAc, 5 mM MgCl2, 50 mM Tris, pH 7.4, and 4 mM dithiothreitol, layered over 2 ml of the same buffer containing 0.5 μM sucrose, and RNCs were isolated by centrifugation in a SW55 rotor (Beckman Instruments, Palo Alto, CA) at 46,000 rpm for 90 min at 4 °C. RNC pellets were resuspended in ribosome binding buffer (RB, 50 mM Tris, pH 7.4, 100 mM KOAc, 10 mM MgCl2, 4 mM dithiothreitol, and 1.5 units/ml RNasin) and then treated by centrifuging at 14,000 × g for 5 min to remove insoluble materials. Ribosome concentration was determined by using a micromolar extinction coefficient of 60.8 at 260 nm, calculated using 4.5 × 10^6 Da as the molecular mass for the 80 S ribosome and an A260 of 13.5 for a 0.1% solution of ribosomes (28). Ribosomes were brought to 1–2 mg/ml with RB. Ribosome-luciferase complexes (ribo-Luc) consisting of the first 86 residues of luciferase (Luc) were synthesized as above using the T7 luciferase control plasmid (Promega) that was restricted in the coding region with Cfr101 (19). RNCS were used immediately, or aliquoted, flash frozen in liquid nitrogen, and stored at −70 °C. Binding reactions were nearly identical for fresh and previously frozen RNCs (data not shown). RNC preparations were used fresh and previously frozen RNCs (data not shown). RNC preparations were used fresh and previously frozen RNCs (data not shown). RNC preparations were used fresh and previously frozen RNCs (data not shown). RNC preparations were used fresh and previously frozen RNCs (data not shown). RNC preparations were used fresh and previously frozen RNCs (data not shown).

To examine if RNC binding is saturable, binding assays were performed as above except that the RNC concentration was varied from 13 to 424 nM while keeping all other conditions constant. The disintegration/min of RNC-mitochondria complexes was measured and the number of moles of RNCs bound was calculated using the following assumptions: 1) each RNC was loaded with a complete nascent chain; 2) there are 260 methionines in the mMDH-met6 nascent chain (ribo-Met); 3) the specificity of the [35S]methionine was 1,000 Ci/mmole; 4) the radiolabeled methionine concentration in the translation reaction was 0.61 μM and the unlabeled methionine concentration was 3.79 μM; and 5) 85% of the radioactivity in RNC preparations was contained within a ribosome-associated nascent chain (see Fig. 1C). The amount of unlabeled methionines in the translation reaction was determined using the concentration of unlabeled methionines in the Redivue [35S]methionine (4.9 μM at 1,000 Ci/mmole and 15 mCi/ml) and the endogenous methionine concentration in the rabbit reticulocyte lysate (5 μM) (29). One-site binding analysis (y = ax/b + x) was performed using Sigma Plot 5.0 software to calculate binding maximality and the effective concentration of the binding assay (x). By omitting mitochondria from the binding assay, background binding was determined to be ~1% of the total (data not shown).

**Effects of Various Treatments in the Binding of RNCs to Mitochondria**—To determine the contributions of the ribosomal component of RNCS in binding, competition binding assays were performed where the bindings were designated to mitochondria with increasing amounts of non-translating ribosomes (0–516 nM). Competition assays were also performed with mitochondria that were pretreated with 5 μg/ml proteinase K using non-translating ribosome concentrations of 0–483 nM. Hyperbolic decay analysis (y = (ab)/(bx + a)) was used to calculate the concentration necessary to inhibit one-half of RNC binding. Analysis by Sigma Plot 5.0 software calculated using y = x/(a + bx). Ribosome binding to proteins on the outer mitochondrial membrane was assessed by subjecting mitochondria to limited proteolysis and then assaying ribosome binding. Mitochondria (10 mg/ml) were treated with...
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**RESULTS**

**Creating Ribosome-mMDH-Methionine<sub>6</sub> Complexes**—The extensive studies investigating RNC targeting to ER were used as a guide in developing assays to investigate ribosome-mitochondria interactions. As these studies have shown, stable RNCs represent the initial targeting stages of the proteins that are targeted to, and imported across, membranes co-translationally (15, 17, 19). Nascent chains of ∼90 residues were used so that 50 residues will be exposed outside the ribosomal structure (33), which is sufficient to span both the inner and outer mitochondrial membranes (34). We enhanced the mMDH nascent chain signal by adding 6 methionines to the carboxyl terminus because wild-type mMDH produced a weak signal in our binding assays. Fig. 1A is a schematic of a wild-type mMDH nascent chain and a mMDH-M<sub>6</sub> nascent chain, which consists of the first 83 amino acids of mMDH, followed by a 4-residue linker and 6 methionines. Fig. 1B is a phosphorimage of ribo-mMDH-M<sub>6</sub> that was separated by SDS-PAGE. As predicted, there are primarily two radiolabeled species that appear when RNCs are analyzed by SDS-PAGE: the nascent chain (10 kDa) and the peptidyl-tRNA (34 kDa). When ribo-mMDH-M<sub>6</sub> was treated with 1 mM puromycin, the entire radioactive signal migrated at 10 kDa indicating that the 34-kDa band is because of the covalent linkage of the tRNA to the nascent chain. This is consistent with the finding that when ribosomes come to the end of a mRNA that lacks a stop codon, the tRNA remains covalently linked to the nascent chain (26, 27). Ribo-Luc behaved in the same manner (data not shown). It was not necessary to increase the Luc nascent chain signal because it contains 4 methionines that can be radiolabeled. To ensure that the radioactive signal of RNC preparations actually represented ribosomes loaded with a nascent chain, either ribo-

proteinase K (0.1–10 µg/ml) for 15 min at 22 °C, and the reaction was inhibited with 2 mM Pefablock (Roche Applied Science) and incubated on ice for 5 min. As a control, one sample of mitochondria was put through the same conditions, except that proteinase K was omitted. Mitochondria were isolated by centrifugation at 8,000 × g for 10 min, washed twice in MIB to remove excess proteinase K and Pefablock, and resuspended in MIB in a volume equal to the protease reaction. Mitochondria were immediately used for the binding assays described above.

To determine the contribution of cations on RNC binding, RNC-mitochondria complexes were prepared under the conditions described above, except that the reaction was scaled up to a final volume of 280 µl. After forming RNC-mitochondria complexes, the reaction was separated into 12 20-µl samples and the complexes were pelleted at 8,000 × g for 10 min at 4 °C. To determine the effect of KCl on binding, the resulting pellets were resuspended in 100 µl of binding buffer containing increasing concentrations of KCl (0–500 mM) and incubated at 22 °C for 5 min. The remaining RNC-mitochondria complexes were isolated by centrifugation at 8,000 × g for 10 min, resuspended, and the remaining binding was determined. To determine the contribution of magnesium cations, RNC binding was assessed by performing the above assay for KCl, except that magnesium chloride was omitted from the resuspension buffer.

**Sucrose Gradient Analysis of RNCs under Various Conditions**—Either ribo-mMDH-M<sub>6</sub> or ribo-Luc (30 µg) was diluted in normal RB or RB that was supplemented with either 300 mM KCl (high salt) or 20 mM EDTA (minus magnesium) in a final volume of 400 µl. The samples were incubated at 22 °C for 5 min followed by 10 min on ice, layered over 4.8 ml of a 10–40% linear sucrose gradient containing the same buffer as the incubation, and centrifuged in an SW55 rotor (Beckman Instruments) at 46,000 rpm for 90 min at 4 °C. Ten 500-µl fractions were collected and the A<sub>260</sub> and counts/min were measured for each fraction. The counts/min measurements between experiments were normalized by calculating the relative counts/min in each fraction compared with the total within each experiment.

**Fig. 1. Construction of mMDH-M<sub>6</sub> nascent chain.** A, schematic of the wild-type mMDH nascent chain and the mMDH-M<sub>6</sub> nascent chain. The nascent chains are identical for the first 83 residues, including the 24 residues at the amino terminus that acts as the mitochondrial targeting sequence (MTS). In addition to the 83 amino-terminal residues from the wild-type mMDH precursor protein, the mMDH-M<sub>6</sub> nascent chain also contains a 4-residue linker (shaded region) followed by the 6 methionines (vertical lines). The nascent chains in RNCs are covalently linked to a tRNA at the carboxyl terminus. B, ribo-mMDH-M<sub>6</sub> analysis. Ribo-mMDH-M<sub>6</sub> was produced in the presence of [35S]methionine as described under “Experimental Procedures,” and equal amounts (∼5 µg of ribo) were either treated, or untreated with 1 mM puromycin for 15 min on ice, separated via SDS-PAGE and the signals were captured using a phosphorimager. C, sucrose gradient analysis of RNCs. Ribo-mMDH-M<sub>6</sub> or ribo-Luc (30 µg) were layered over a 10–40% sucrose gradient, separated, and fractions were collected as described under “Experimental Procedures.” The absorbance at 260 nm (∙) and counts/min (○) were measured for each fraction. n = 3 for each data point.
Evidence of a Ribosome Receptor on Mammalian Mitochondria

Mechanism of RNC Binding to Mitochondria—Studies have shown that ribosomes actively translating proteins interact with both protein and lipid components of isolated microsomes. The signal sequence interacts with both membrane lipids and the proteins of the translocon machinery, and the ribosomes bind directly to the proteins of the translocon (35–40). We predicted that RNCs would interact with mitochondria in a similar manner through a number of different types of interactions including: 1) interaction with one or more specific proteins in the membrane that could act as a ribosome receptor; 2) interaction of the nascent chain with specific membrane proteins, including the already described receptors used in the post-translational import of proteins; 3) nonspecific electrostatic interactions between charged/polar membrane lipids or proteins and charged/polar groups on the ribosome or nascent chain; 4) a combination of any of these interactions. We used a novel binding assay, which specifically follows the binding of ribosomes that are loaded with a known nascent chain; 4) a combination of any of these interactions. We used a novel binding assay, which specifically follows the binding of ribosomes that are loaded with a known nascent chain.

To determine the contribution of the ribosomal component of the RNC in binding, a competition assay was performed to determine whether non-translating ribosomes compete with RNCs for binding sites on mitochondria. For these experiments, 80 S ribosomes were isolated from nuclease-treated rabbit reticulocyte lysate and used to compete RNCs. We found that RNCs have a competitive binding advantage over non-translating ribosomes, regardless of whether the nascent chain was for a mitochondrial protein or not (Fig. 3). Regression analysis indicated that it required more than two times the

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Interestingly, ribo-mMDH-Met6 was significantly protease-insensitive binding was also specific to the ribosome indicated amounts of non-translating ribosomes (0–516 nM) were simultaneously added to mitochondria and RNC binding was determined as described under “Experimental Procedures.” The data represents the averaged data from at least three experiments with the data standardized by setting the value of ribosome binding without non-translating ribosomes to 1.0. The data were subjected to a hyperbolic decay analysis, which calculated a concentration to inhibit binding by one-half (IC50) for non-translating ribosomes of 167 ± 23 nM for ribo-mMDH-Met6 (r² = 0.92) and 160 ± 19 nM for ribo-Luc (r² = 0.95).

The fact that RNC binding persists at high concentrations of non-translating ribosomes to inhibit half of the RNC binding for both RNC species. However, regression analysis that included a non-inhibitable function predicted that only ribo-Luc would be eventually displaced by an excess of non-translating ribosomes and that ~20% of ribo-mMDH-Met6 binding is independent of the ribosome. These data indicate that while the nascent chain enhances the binding of both RNC species and is directly involved in ribo-mMDH-Met6 binding, the primary binding component of the RNC is the ribosome.

To determine whether RNCs bind through proteins located on the mitochondrial surface, we lightly pretreated mitochondria with increasing amounts of proteinase K to degrade proteins on the outer membrane. When proteinase K-treated mitochondria were used for binding assays, RNC binding decreased as proteinase K treatment increased with half of the protease-sensitive binding being inhibited at 1 μg/ml (Fig. 4A). Binding continued to decrease as proteinase K treatment increased until treatment exceeded 5 μg/ml, where about 40% of the RNCs remained bound. As before, there was no significant difference in the binding of ribosomes loaded with either mMDH-Met6 or -Luc nascent chains. These findings indicate that there are protease-sensitive proteins on the mitochondrial surface that participate in RNC binding. Binding inhibition was not a result of disrupting the integrity of the outer membrane because the proteinase K treatments had no significant effect on the respiratory control ratio as determined with a Clark electrode (data not shown), which is consistent with previous findings (41).

The fact that RNC binding persists at high concentrations of proteinase K treatment (10 μg/ml) suggests that binding also occurs through other interactions. Using the non-translating ribosome competition assay described above with mitochondria pretreated with 5 μg/ml proteinase K, we determined that the protease-insensitive binding was also specific to the ribosome (Fig. 4B). Interestingly, ribo-mMDH-Met6 was significantly less sensitive to lower concentrations of non-translating ribosomes than ribo-Luc. We also observed a difference in how each RNC species bound compared with normal mitochondria in the presence of non-translating ribosomes. There was no statistical difference in the inhibitory effect of non-translating ribosomes on ribo-Luc binding to proteinase K-treated mitochondria compared with normal mitochondria, whereas non-translating ribosome inhibition of ribo-mMDH-Met6 binding was significantly less when using proteinase K-treated mitochondria. The data indicates that proteinase K-insensitive binding also occurs through the ribosome for both RNC species, and in support of this finding in Fig. 3, a portion of the rbo-mMDH-Met6 binds through a mechanism other than the ribosome.

The contribution of electrostatic interactions in RNC binding was assessed by incubating preformed RNC-mitochondria com-
plexes in binding buffer that contained increasing amounts of KCl (0–500 mM). RNC binding decreased as a function of increasing KCl concentration with half of the total RNCs being released when the resuspension buffer contained KCl concentrations just below 200 mM and 75% being released at 300 mM (Fig. 5A). There was no difference in how the various KCl concentrations affected the binding of RNCs programmed with either mMDH-Met6 or +Luc nascent chains. RNC-mitochondria complexes resuspended in buffer lacking KCl remained tightly associated with mitochondria (<5% released of total), indicating that the isolation and resuspension procedure did not disrupt RNC-mitochondria interactions. To determine the possible effects that high concentrations of KCl had on ribosomal structure, RNCs were analyzed by sucrose gradients after incubating in 300 mM KCl (Fig. 5B). There was a slight shift in the ribosome sedimentation profile compared with Fig. 1C, but when compared with Fig. 5C, where the subunits were completely dissociated, it is clear that the subunits were still associated and the nascent chain was still associated with the ribosome. Roughly 15% of the RNCs remained bound at 0.5 M KCl concentrations, indicating that while most of the RNC binding is not stable under high ionic conditions, other factors minimally contribute to binding.

Other investigators have found that ribosomal structure and function is dependent on magnesium concentration (42, 43). We determined that RNC binding is extremely sensitive to the lack of MgCl2 by performing the KCl assay as before, except that MgCl2 was omitted from the resuspension buffer (Fig. 5A). RNC-mitochondria interactions were very stable in the presence of MgCl2 when no KCl was present (5% released), but roughly 50% of the RNCs were released by incubating RNC-mitochondria complexes in a buffer lacking both MgCl2 and KCl. The release of RNCs as a function of increasing KCl concentration was also very different in the absence of magnesium; binding sharply decreased in a hyperbolic manner as KCl concentration increased compared with the sigmoidal manner when magnesium was present. When magnesium was removed from RNCs by EDTA treatment and then analyzed by sucrose gradients, we found that RNCs dissociate into their subunits and release their nascent chain (Fig. 5C). These findings show that RNC binding is dependent on an intact ribosomal structure.

**DISCUSSION**

Ribosomes actively synthesizing nascent polypeptides are targeted to the ER (1), the outer and inner mitochondrial membranes (3–8, 15, 19, 44), the bacterial inner membrane (45), and the thylakoid membranes in plant chloroplasts (46). Although ribosome binding to the mitochondrial outer membrane has been known for years, few studies have focused on determining the mechanism of this interaction. In addition, most of the studies investigating mitochondrial protein import have been performed in yeast and may not represent how proteins are targeted to mammalian mitochondria because of the differences in the transmembrane protein transfer that exist between yeast and mammalian cells (47). By following the binding patterns of mammalian ribosomes loaded with a known nascent chain, we have investigated the mechanism of ribosome binding to mammalian mitochondria.

We found that RNC binding to the outer mitochondrial membrane has both similarities and differences with binding to other cellular membranes. If mitochondria possess specific binding site(s) for RNCs like those in the ER where they bind specific proteins in the membrane, the binding capacity of mitochondria should be limited. Indeed, we found that RNC binding to mitochondria is saturable, which is in agreement with our previous finding that non-translating ribosome binding is also saturable (19). Because there appeared to be specific

![Fig. 5. RNC binding is sensitive to ionic strength and magnesium.](http://www.jbc.org/)

**A**. RNC-mitochondria complexes were formed and isolated using either ribo-mMDH-Met6 (○) or ribo-Luc (△), as described under “Experimental Procedures.” The complexes were then resuspended in the same buffer as the binding reaction, except it contained varying amounts of KCl (0–500 mM) and MgCl2 was either present at 10 mM or omitted completely. RNC-mitochondria complexes were re-isolated and the remaining RNC binding was determined. The data are presented as the amount of counts/min released/total counts/min for each individual sample and represents the averaged data from at least three experiments. B and C, sucrose gradient analysis of RNCs in various cation conditions. Ribo-mMDH-Met6 or ribo-Luc (30 μg) were treated with either 300 mM KCl (B) or 20 mM EDTA (C) as described under “Experimental Procedures.” Samples were then layered over a 10–40% sucrose gradient, separated, and fractions were collected as before, and the absorbance at 260 nm (□) and counts/min (●) was measured for each fraction. n = 3 for each data point.
binding sites on mitochondria for RNCs, we determined the contribution of the ribosomal component of the RNC in binding. We reasoned that if binding were solely dependent on the ribosome, non-translating ribosomes would effectively compete for the binding of RNCs. Instead, we found that non-translating ribosomes were able to compete for binding, but only poorly. This suggests that the ribosome is the main binding component of the RNC and other portions act to stabilize binding. It is possible that the nascent chain or associated cytosolic chaperones are responsible for this stabilization, but further experiments will be needed to determine this. In addition, only RNCs loaded with Luc can be completely inhibited by an excess of non-translating ribosomes, suggesting that the mMDH nascent chain may engage components of the mitochondria independent of the ribosome. However, the contribution of the mMDH nascent chain in ribo-mMDH binding is much less than the ribosome because the amount of non-translating ribosomes necessary to inhibit binding by one-half is the same for ribo-Luc. These data are consistent with our previous finding that the mMDH nascent chain alone is inadequate to target ribosomes to mitochondria in conditions that inhibit the binding of non-translating ribosomes and suggests that specific ribosomal binding sites exist on the mitochondrial outer membrane (19).

Ribosome binding has been evolutionarily conserved in bacterial and ER membranes with the 28 S rRNA of the ribosomal large subunit directly interacting with the proteinaceous translocation pores (21). By lightly pretreating mitochondria with protease, we determined that a majority of the RNCs bind via proteins located on the outer membrane. Competition assays involving non-translating ribosomes indicate that the protease-insensitive binding is also specific for ribosomes, although it is unclear whether it is through lipids or protease-insensitive proteins. This is similar to ribosome binding to the ER, where the membrane components involved are also protease-sensitive, but proteolysis of the main binding site, Sec61, does not inhibit all binding if other components are intact (35, 48, 49). It has also been shown that RNCs can non-specifically bind protein-free lipid bilayers (40). In support of the finding above that mMDH nascent chains bind independently of the ribosome, we found that ribo-mMDH is less sensitive than ribo-Luc to non-translating ribosome inhibition when binding protease-treated mitochondria. The protease treatment that decreased RNC binding by 60% most likely removed outer membrane components that interact with the ribosome, allowing the contribution of the mMDH nascent chain to be greater than it was when binding normal mitochondria. The nature of the mMDH nascent chain interaction with protease-treated mitochondria is not clear, but it is possible that the ribosome is acting as a chaperone to deliver the mMDH nascent chain to the Tom40 translocation pore, which contains protease-insensitive binding sites specific for precursor proteins (50). Together, these data suggest that RNC binding occurs via multiple steps involving several membrane components.

Cations play a crucial role in regulating the electrostatic forces that govern protein-protein and protein-nucleic acid interactions. Although RNCs tightly bind mitochondria in conditions of low ionic strength, we found that binding decreases as KCl concentration is increased indicating that binding is primarily through electrostatic interactions. This electrostatic interaction is characteristic of typical receptor-ligand interactions. The midpoint of RNC release as a function of KCl concentration is slightly above the intracellular potassium concentration, supporting the physiological significance of RNC binding to mitochondria. Maximal dissociation was observed at KCl concentrations greater than 300 mM, which is consistent with the finding that yeast mitochondria do not bind ribosomes at 350 mM KCl (4). This sensitivity to high salt differs from ribosome binding to ER and mitochondrial inner membranes, where only about half of the ribosomes are released at 500 mM KCl (44, 51). The salt-insensitive binding to ER, but not mitochondrial inner membranes, is through the nascent chain, exemplifying the differences that exist between co-translational systems.

We found that RNC binding is also highly dependent on the presence of magnesium cations, which is consistent with the finding that ribosome binding to yeast mitochondria decreases as magnesium concentration is lowered (6). Interestingly, not only does the lack of magnesium inhibit binding, it also amplified the inhibitory effect of KCl. This is most likely because of the vital role that magnesium plays in ribosomal structure. Consistent with previous reports that ribosomes completely dissociate when treated with 200 mM KCl in the absence of magnesium (52), we found that removing magnesium from the RNCs caused them to dissociate into subunits and release the nascent chain. These data suggest that RNC binding to mitochondria is dependent on an intact ribosomal structure.

Although the presence of a nascent chain had an effect on RNC binding, we did not observe a difference in the binding patterns of ribosomes loaded with mMDH or Luc nascent chains in most of the experiments performed. This was not surprising because even though we had previously reported that mitochondria use GTP to distinguish between ribo-mMDH and ribo-Luc, ribo-Luc was still able to bind (19). These experiments were designed to investigate how ribosomes bind to mitochondria, which represents an earlier event than mitochondrial protein import. The data presented here suggest that it is the ribosomal component of the RNC, not the nascent chain, which mainly interacts with mitochondria. This is similar to signal recognition particle-independent RNC binding to the Sec61 complex in ER, which is also not dependent upon the signal sequence and binding of the nascent chain is independent of ribosome binding (31, 53). Taken together, our data indicate that RNCs specifically bind mammalian mitochondria via electrostatic interactions with proteins on the outer membrane. This supports the hypothesis that the outer membrane of mitochondrial mitochondria contains specific receptors for ribosomes.

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