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Fyn Kinase Regulates Translation in Mammalian Mitochondria

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Abstract

Background—Mitochondrial translation machinery solely exists for the synthesis of 13 mitochondrially-encoded subunits of the oxidative phosphorylation (OXPHOS) complexes in mammals. Therefore, it plays a critical role in mitochondrial energy production. However, regulation of the mitochondrial translation machinery is still poorly understood. In comprehensive proteomics studies with normal and diseased tissues and cell lines, we and others have found the majority of mitochondrial ribosomal proteins (MRPs) to be phosphorylated. Neither the kinases for these phosphorylation events nor their specific roles in mitochondrial translation are known.

Methods—Mitochondrial kinases responsible for phosphorylation of MRPs enriched from bovine mitoplasts by strong cation-exchange chromatography and identified by mass spectrometry-based proteomics analyses of kinase rich fractions. Phosphorylation of recombinant MRPs and 55S ribosomes was assessed by *in vitro* phosphorylation assays using the kinase-rich fractions. The effect of identified kinase on OXPHOS and mitochondrial translation was assessed by various cell biological and immunoblotting approaches.

Results—Here, we provide the first evidence for the association of Fyn kinase, a Src family kinase, with mitochondrial translation components and its involvement in phosphorylation of 55S ribosomal proteins *in vitro*. Modulation of Fyn expression in human cell lines has provided a link between mitochondrial translation and energy metabolism, which was evident by the changes in 13 mitochondrially encoded subunits of OXPHOS complexes.

Conclusions and General Significance—Our findings suggest that Fyn kinase is part of a complex mechanism that regulates the protein synthesis and OXPHOS possibly by tyrosine phosphorylation of translation components in mammalian mitochondria.

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Keywords

Fyn Kinase; Tyrosine Phosphorylation; Mitochondrial Ribosomal Proteins (MRPs); Mitochondrial Protein Synthesis (Translation)

1. Introduction

Mitochondrial translation is responsible for the synthesis of 13 mitochondrially-encoded subunits of oxidative phosphorylation (OXPHOS) complexes, which generate approximately 90% of the energy for a eukaryotic cell in the form of ATP. In addition to the changes and defects in expression of mitochondrial ribosomal proteins (MRPs) and translation factors, post-translational modifications (PTMs) of these translation components are important for the regulation of OXPHOS in mammalian mitochondria [1–4]. Our laboratory has previously shown that MRPs are modified by phosphorylation and acetylation to regulate the synthesis of these 13 essential proteins [1, 4]. In the phosphoproteomic analysis of bovine 55S ribosomes, we identified at least twenty-four phosphorylated MRPs at Ser/Thr or Tyr residues [1, 2]. Since the initial discovery of phosphorylation of MRPs, high throughput analyses of phosphoproteomes of normal and diseased tissues and cell lines revealed that the majority of MRPs were phosphorylated [5–9]. Interestingly, the phosphorylation sites in these proteins are mostly located in functionally significant regions of the ribosome, possibly involved in regulation of the function of these sites in protein synthesis and energy metabolism in health and disease [2, 10]. In fact, phosphorylation of MRPS29, also known as DAP3, is critical for its role in induction of apoptosis in various cell lines [2, 11]. Changes in the expression and phosphorylation of DAP3 in various cancer cells and tumors suggest a significant role for this modification in disease states [11–13]. However, the investigation of the role of a specific phosphorylation instance in MRPs is a challenging task because they are part of a large ribonucleoprotein complex.

One of the current approaches to study dynamic changes of PTMs and their role in regulation of multi-subunit complexes such as ribosomes is to identify the enzyme(s) responsible for these PTMs. With this approach, we have identified the ribosome-associated SIRT3 as the NAD⁺-dependent deacetylase responsible for the deacetylation of MRPL10 and shown its role in protein synthesis [4]. Similarly, several Ser/Thr kinases and members of the Src family tyrosine kinases (SFKs) have emerged as major regulators of energy metabolism in mammalian mitochondria in health and disease [14, 15]. Although localization of Ser/Thr kinases into the mitochondria is not clear, SFK members such as cSrc and Fyn were shown to translocate into the mitochondria and phosphorylate subunits of complex I, II, and IV and pyruvate dehydrogenase in mammals [16–19]. In a recent study, Matsushima *et al.* reported that Fyn kinase and NOX4 are co-located in mitochondria and regulate ROS production and cell death in cardiomyocytes [20]. Specifically, the Tyr phosphorylation of pyruvate dehydrogenase by SFKs was shown to be one of the key regulators of this complex in promoting the Warburg effect in various cancers [19]. These observations are also supported by the inhibition of OXPHOS process in osteosarcoma and prostate cancer cell lines treated with the SFK inhibitors [21, 22].

In this study, we provide the first evidence for a kinase responsible for phosphorylation of MRPs and 55S ribosomes *in vitro*. Fractionation by strong cation exchange (SCX) chromatography of highly purified mitoplasts has resulted in a fraction with high kinase activity. Protein identification in this fraction by LC-MS/MS revealed the presence of Fyn kinase associated with this high kinase activity. Furthermore, the detection of MRPs and translation factors in the same fraction supported the role of this kinase in the regulation of mitochondrial translation by phosphorylation. siRNA-directed knock-down of Fyn kinase in human cell lines reduced the expression of a mitochondrially encoded subunit of complex IV, while the over-expression stimulating the synthesis of this subunit. These observations led us to propose that the Fyn-dependent phosphorylation of mitochondrial translation components is involved in regulation of OXPHOS and energy metabolism in mammalian mitochondria.

2. Materials and Methods

2.1 Isolation of Bovine Liver Mitoplasts and Mitochondrial Ribosomes

Preparation of bovine mitoplasts (mitochondria without outer membrane) and mitochondrial ribosomes starting from 4 kg of bovine liver was adapted from previously described methods[23–25]. To preserve protein phosphorylation, phosphatase inhibitors (2 mM imidazole, 1 mM sodium orthovanadate, 1.15 mM sodium molybdate, 1 mM sodium fluoride, and 4 mM sodium tartrate dehydrate) were added to mitochondrial lysates prior to the SCX fractionation and ribosome purification.

2.2 Strong Cation Exchange (SCX) Enrichment of Mitochondrial Kinase Activity

Mitoplasts from bovine liver were lysed in 0.26 M sucrose, 50 mM KCl, 15 mM MgCl₂, 20 mM HEPES pH 7.6, 1 mM EDTA, 0.05 mM spermine, 0.05 mM spermidine, 1% Triton X-100, as well as protease and phosphatase inhibitors. The lysate was incubated at 4 °C for 20 min, followed by centrifugation at 15,000 rpm for 45 min at 4 °C. The supernatant was dialyzed in 50 mM KCl, 5 mM MgCl₂, 20 mM HEPES pH 7.6, 1 mM EDTA, and 10% glycerol at 4 °C. The dialyzed sample was loaded on a column prepared with SP Sepharose Fast Flow resin (Amersham Biosciences Inc.), washed twice with 50 mM KCl, 5 mM MgCl₂, 20 mM HEPES pH 7.6, and 1 mM EDTA, and eluted with 0.05–1.5 M KCl in a step-wise manner. Each wash and elution contained protease and phosphatase inhibitors. Aliquots were taken and tested for the presence of kinase activity by incubating with and without 0.3 µg of recombinant proteins MRPs, 100 µM cold ATP, and 2.5 µCi [γ -³²P] ATP for 1 hr at 30 °C followed by SDS-PAGE and autoradiography. Purified 55S bovine mitochondrial ribosomes were also treated similarly *in vitro* phosphorylation assays as described before [1]. Expression and purification of recombinant MRPS18B, MRPL10, MRPL11, and MRPL12 were described previously [26]. SCX column fractions with the greatest kinase activity were acetone precipitated and separated on a large 10% SDS-PAGE gel for protein identification by mass spectrometry.

2.3 Protein Identification by Mass Spectrometry

For protein identification by tandem mass spectrometry, tryptic digests of protein bands were analyzed by a nano cap-LC-MS/MS setup that consisted of a Surveyor HPLC pump, a

Surveyor Micro AS autosampler, and an LTQ linear ion trap mass spectrometer (ThermoFinnigan Co.). Tandem MS spectra obtained by fragmenting a peptide by collision-induced dissociation (CID) were processed and analyzed using Xcalibur 2.0 and Bioworks 3.2 software. The raw CID tandem MS spectra were also converted to Mascot generic files (.mgf) using the extract msn software (ThermoFinnigan Co.). The .dta and .mgf files were submitted to site-licensed Mascot (version 2.2) search engines to search against an in-house bovine protein sequence database extracted from UniProtKB database. Database searches were performed with cysteine carbamidomethylation as a fixed modification for cases in which cysteines were alkylated by iodoacetamide. The variable modifications were methionine oxidation (+16 Da) and phosphorylation (+80 Da) of Ser, Thr, and Tyr residues and loss of water (−18 Da) from Ser and Thr due to β -elimination during loss of the phosphate moiety. Up to 2 missed cleavages were allowed for the protease of choice. Precursor and fragment mass tolerances were set to 3 and 2 Da, respectively. Tandem MS spectra that matched to a sequence were manually evaluated at the raw data level with consideration of overall data quality, signal-to-noise of matched peaks, and the presence of unmatched dominant peaks to ensure data quality.

2.4 Immunoblotting Analysis

Protein concentration was determined by BCA assay (Pierce, Rockford, USA) using bovine serum albumin (BSA) as a standard. Protein lysates were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad, Richmond, USA). The membranes were blocked in 5% (w/v) dry skim milk powder and 1% BSA, dissolved in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST), washed with TBST three times, and incubated with the corresponding primary antibody overnight at 4 °C. The antibodies for OXPHOS subunits were obtained from Abcam (Cambridge, USA). The Fyn, MRPS29, and MRPL11 antibodies were purchased from Cell Signaling Technologies Inc. The rabbit anti-MRPL47 was generated by Covance Inc. The secondary antibodies, mouse and rabbit IgG HRP conjugates (Pierce, Rockford, USA), were all used for 1 h at a 1:3,000 dilution. The immunoreactivity of the proteins was detected using the ECL chemiluminescent kit from Amersham (GE Healthcare, USA). The membranes were developed using the protocols provided by the manufacturer.

2.5 Cell Culture

Monolayer cultures of HepPG2, Hep3B, and HEK293T cell lines obtained from ATCC were maintained in DMEM medium (HyClone, Thermo-Scientific) adjusted to contain 1g/L glucose, 4 mM glutamine, 2 mM pyruvate, 10% fetal bovine serum (FBS) (HyClone, Thermo-Scientific) and 1% penicillin-streptomycin (P/S) (Corning Cellgro, Manassas, VA). The cells were grown in a humidified incubator at 37 °C and 6% CO₂. Transfections of siRNAs and plasmid vectors were performed using the Mirus transfection reagent according to the manufacturer's protocols (Mirus Bio LLC). Partial knock-down and ectopic expression of human Fyn were performed using Fyn and control siRNAs and the vectors were obtained from Santa Cruz Biotechnology Inc. and Addgene, respectively. pRK5-cFyn plasmid was deposited to Addgene by Dr. Filippo Giancotti and described previously [27].

2.6 [³⁵S]-Methionine Pulse Labeling of Mitochondrial Translation Products *In Vivo*

Pulse labeling experiments were performed in minimum essential DMEM medium without methionine and cysteine and dialyzed serum (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 10 mM KCl) as indicated in previous reports [4, 32]. Cytoplasmic protein synthesis was arrested by incubating cells with emetine-containing medium for 15 min. To label the mitochondrially-encoded proteins, 0.2 mCi/mL of [³⁵S]-Methionine and Cysteine mixture (Perkin Elmer) was added to the cells. After 2 h incubation, cells were lysed in 1× cell lysis buffer (Cell Signaling Technologies). Electrophoresis of whole cell lysates (30 µg) was performed through 12% SDS-PAGE. The gels were dried and exposed to autoradiography films. The bands intensities were quantified by Un-Scan-It.

2.7 Mitochondrial Complex IV Activity Assay

HepG2 and Hep3B cell pellets were dissolved in a 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 100 µM reduced cytochrome *c*. The complex IV enzymatic activity assay was performed by monitoring the oxidation of cytochrome *c* at 550 nm as described previously [53]. The rate is calculated by dividing the absorbance difference between two linear points by the time point difference (Rate = (Abs 1 – Abs 2)/(Time 2 – Time 1)).

3. Results and Discussion

3.1 Enrichment of kinase-rich fractions and phosphorylation of recombinant MRPs

The lysate of bovine mitoplasts was fractionated on an SCX column to identify kinases responsible for phosphorylation of mitochondrial translation components, mainly MRPs. The steps used in the mitochondrial kinase enrichment and identification are given in Fig. 1A. SDS-PAGE analysis of proteins eluted from the SCX column indicated that the majority of the proteins retained were eluted at KCl concentrations greater than 0.5 M (data not shown). This observation is in agreement with the fact that the SCX resin retains highly basic proteins.

Removal of high salt concentrations in SCX fractions by dialysis was followed by kinase activity assays. The fractions with high kinase activity contained several phosphorylated MRPs. Among them were the L7/L12 stalk proteins, MRPL10, MRPL11, and MRPL12 and a small subunit protein MRPS18B of the 55S ribosome [24, 25, 28]. A mixture of these recombinant proteins was tested in *in vitro* phosphorylation assays using the SCX fractions for a quick scan of kinase activity (data not shown). Two of the fractions with higher kinase activities, eluted at 0.75 and 1M KCl, were used in phosphorylation of recombinant MRPs (Fig. 1B). Along with several endogenous phosphorylated proteins, MRPL10, MRPL11, and MRPS18B were phosphorylated in the presence of [³²P]-ATP (Fig. 1B). Phosphorylation of MRPL11 and MRPS18B was more prevalent in assays performed with the 1M fraction implying the specificity or the amount of the kinase present in this fraction (Fig. 1B). In addition to the differences observed in phosphorylation of recombinant MRPs, endogenous protein phosphorylation patterns were different in the control lanes for these two fractions (Fig. 1B), suggesting either the presence of different kinases or substrates.

3.2 Identification of Fyn kinase and mitochondrial translation components in kinase-rich fractions

In vitro phosphorylation of MRPs with two kinase-rich fractions led us to the identification of proteins found in these fractions by tandem mass spectrometry. The kinase-rich fractions containing 0.75 and 1M KCl were separated on 10% SDS-PAGE. The protein bands between 34 and 110 kDa were excised and digested with trypsin (Fig. 2A). Proteins identified in the analyses of tryptic peptides by nano-LC-MS/MS are listed in the supplemental Table S1. Among the proteins identified were only two kinases, pyruvate dehydrogenase kinase (PDK) and Fyn kinase. The majority of the proteins identified were from the large mitochondrial metabolic complexes such as pyruvate and alpha-ketoglutarate dehydrogenases and ribosomes (Table S1). This finding was extremely important since the kinase-rich fractions were also active in the phosphorylation of recombinant MRPs shown in Fig. 1B. The other mitochondrial translation components including elongation factors Tu (mtEF-Tu) and Ts (mtEF-Ts) and tRNA synthetases were also identified as a major group of proteins (Table S1).

PDK is a Ser/Thr kinase responsible for the regulation of the PDH complex by phosphorylation of the PDHA1 subunit and the majority of this kinase was found in the 0.75 M fraction along with its substrate, PDHA1 (Fig. 1B and Table S1). The dominant kinase identified in the 1M fraction with three peptides was Fyn kinase (Fig. 2B). Fyn is a Tyr kinase, which belongs to the SFKs with a highly similar primary sequence to Src and Yes kinases. However, the alignment of bovine Src, Fyn, and Yes kinases and the uniqueness of two of these peptides detected by mass spectrometry allowed us to identify this protein specifically as Fyn kinase (Fig. 2C).

3.3 Association of Fyn kinase with mitochondrial ribosomal proteins and their phosphorylation *in vitro*

As described above, Fyn kinase was eluted with large and highly positively charged complexes including ribosomes even though its calculated pI is 6.2. For this reason, the elution of this protein at high salt concentrations meant that there were strong functional interactions between Fyn kinase and ribosomal proteins and/or other metabolic complexes found in the same fraction. Western blot analyses were performed with anti-Fyn and MRP antibodies to determine the relative distribution of Fyn kinase and MRPs in 0.75 and 1 M fractions. The Fyn kinase was predominantly found in the 1 M fraction along with the endogenous MRPs, MRPL47 and MRPS29 (Fig. 3A), which was in agreement with the mass spectrometry data. This observation confirmed that the Fyn kinase was associated with large complexes in mammalian mitochondria including ribosomes and other translation components.

Earlier studies from our laboratory revealed that the MRPs of the mammalian mitochondrial ribosome (55S) are phosphorylated at their Ser/Thr or Tyr residues [1]. To investigate the phosphorylation of MRPs as part of the 55S ribosome, the Fyn kinase-rich fraction (1M fraction) was used for *in vitro* phosphorylation of purified bovine 55S ribosomes in the presence of [³²P]-ATP. The 1M kinase-rich fraction resulted in an overall increase in phosphorylation as compared to the control (Fig. 3B). Fyn kinase and PDK found in the

kinase-rich fraction also phosphorylated the PDHA1 subunit of PDH complex migrating at around 45 kDa in the lanes containing the 55S ribosome (Fig. 3B, black arrow). Phosphorylation of PDHA1 in these samples is not unexpected as we demonstrated previously that the PDH complex co-purifies or co-sediments with 55S ribosomes and PDK is tightly associated with the PDH complex and sediments with the ribosomes during the purification [29]. In addition, SFK-dependent phosphorylation of PDHA1 subunit at Tyr301 has been shown previously [30, 31].

3.4 Changes in Fyn kinase expression and its role on synthesis of mitochondrial OXPHOS components

Fyn kinase is one of the highly expressed SFK members in a wide variety of cells and tissues. We evaluated several different human cell lines to knock-down Fyn kinase using siRNAs and study its role on mitochondrial protein synthesis and OXPHOS (data not shown). Human liver HepG2 and HEK293T kidney cell lines were found to be endogenously expressing normal to high level of Fyn kinase. As shown in Figs. 4A and S1A, Fyn kinase expression was significantly reduced both in HepG2 and HEK293T cell lines, respectively, transfected with Fyn specific siRNA relative to the cells transfected with control siRNA as detected by the Fyn antibody. Even though there was slightly more total protein in the Fyn knock-down lane, the majority of OXPHOS components did not change significantly. The only significant difference was observed in the expression of a mitochondrially encoded subunit of complex IV and a nuclear-encoded subunit of complex I, COII and NDUF8 respectively, in cells transfected with the Fyn-specific siRNA (Figs. 4A and S1A). The reduction observed in COII expression is possibly due to the direct effect of Fyn knock-down on mitochondrial translation, while the decrease in NDUF8 is possibly due to the reduction in seven of the mitochondrially encoded subunits of complex I. Considering a higher amount of protein in the Fyn knock-down lane, there was no significant difference in the expression of mitochondrial ribosomal proteins, MRPS29 and MRPL11 (Fig. 4A). In addition to the detection of COII expression by immunoblotting, we performed [³⁵S]-Met pulse labeling of 13 mitochondrial translation products in the presence of cytosolic protein synthesis inhibitor, emetine, as described previously [32]. In consistent with the reduced COII expression in HepG2 and HEK293T cells at steady-state levels, *de novo* expression of 13-mitochondrially encoded was significantly reduced in HEK293T cells transfected with Fyn specific siRNAs (Fig. S1B). Therefore, we suggest that Fyn regulates mitochondrial translation possibly by changing the phosphorylation status of mitochondrial ribosomal proteins and/or translation factors.

In addition to the effect of reduced Fyn kinase expression in cell lines, ectopic expression of Fyn kinase was orchestrated in cell lines with lower endogenous levels of Fyn. Hep3B cell lines were transfected with the c-Fyn kinase expression vector and the expression of Fyn kinase and OXPHOS components were evaluated by immunoblotting analyses. As shown in Fig. 4B, it was possible to overexpress Fyn in Hep3B cell lines. Similar to the knock-down of Fyn, no significant difference was observed in the levels of nuclear encoded subunits of complex V, III, II, and I as well as MRPs (Fig. 4B). On the other hand, the expression of mitochondrially encoded COII subunit of complex IV increased about 50% compared to the cells transfected with the control vector (Fig. 4B). Fyn kinase expression in Hep3B cells also

changed the phosphorylation profile of the proteins compared to the control cells (data not shown).

Differential expression of Fyn kinase in liver hepatoma cell lines, HepG2 and Hep3B, allowed us to compare its role on OXPHOS and synthesis of 13 mitochondrially encoded translation products. While Fyn kinase was ubiquitously expressed in HepG2 cell line, its expression in Hep3B cell line was considerably reduced (Fig. 5A). Interestingly, reduced expression of OXPHOS subunits and complex IV activity coincided with the absence of Fyn kinase in Hep3B cells relative to their expression and activity in HepG2 cells (Figs. 5A and 5B). To determine the effect of endogenous Fyn kinase expression on mitochondrial translation further, [³⁵S]-Met pulse labeling of mitochondrial translation products was performed in HepG2 and Hep3B cell lines. Again, the *de novo* synthesis of 13 mitochondrially encoded subunits of OXPHOS complexes were significantly reduced in Hep3B cells (Fig. 5C). This observation along with the Fyn knock-down data in HepG2 and HEK293T cells (Figs. 4A and S1) supports the idea that Fyn kinase regulate mitochondrial translation and oxidative phosphorylation in mammalian mitochondria.

4. Conclusions

Oxidative phosphorylation is essential for energy generation; however, it is also one of the major contributors of cellular oxidative stress, causing pathological conditions ranging from neurodegenerative disorders to cancer. Therefore, the dynamic and complex set of reversible PTMs of mitochondrial proteins including MRPs are critical in the fine-tuning of energy metabolism. Acetylation and phosphorylation of mitochondrial proteins are the two most abundant PTMs regulating this process [33, 34]. Acetylation so far appears to be mainly regulated by sirtuins. SIRT3, one of the NAD⁺-dependent mitochondrial deacetylases, has multiple targets in mitochondria, and its deficiency has been implicated in many metabolic disorders and cancer [31, 35–40]. Similarly, the kinases responsible for phosphorylation of key metabolic enzymes also regulate energy metabolism in disease states [21, 41–43]. In fact, after our initial reports of MRP phosphorylation in bovine liver mitochondria, phosphorylation of many more MRPs has been reported in high throughput analyses of normal and diseased tissue and cell line phosphoproteomes by LC-MS/MS [5–7, 44]. However, neither the kinases responsible for the majority of MRP phosphorylations nor their effects on mitochondrial translation are known yet.

In the current study, we have demonstrated that Fyn kinase is associated with the mitochondrial translation components and can catalyze the phosphorylation of MRPs *in vitro*. The essential role of Fyn in mitochondrial protein synthesis and OXPHOS has also shown by carrying out siRNA-directed knock-down and ectopic expression of Fyn in human liver cell lines. The reduced expression of Fyn caused a dramatic decrease in mitochondrial translation while its ectopic expression enhanced the mitochondrial translation in these cell lines (Fig. 4). Validation of endogenous Fyn kinase expression and stimulation of mitochondrial translation in cells with higher oxidative phosphorylation capacity is also in agreement with its role in various converging signaling pathways, such as Wnt and insulin signaling to glucose and fatty acid oxidation [45–48].

Our data clearly show that Fyn kinase regulates mitochondrial translation possibly by phosphorylation of its multiple components, and thus, stimulates energy generation in mammalian mitochondria. Fyn-dependent regulation of mitochondrial translation will be play a pioneering role in providing the link between dysregulation of energy metabolism and altered Fyn kinase activities in diseases such as Alzheimer's and cancer [30, 49–52].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Fyn kinase is associated with mitochondrial translation machinery.
2. Mammalian mitochondrial ribosomal proteins are Tyr-phosphorylated by Fyn kinase *in vitro*.
3. Fyn regulates protein synthesis and oxidative phosphorylation in mammalian mitochondria.

A

Fig. 1. Enrichment of mitochondrial kinase(s) responsible for phosphorylation of mitochondrial translation components

A) An experimental scheme used in the enrichment of mitochondrially localized kinase(s).

B) *In vitro* phosphorylation of recombinant mitochondrial ribosomal proteins MRPL12 (L12), MRPL10 (L10), MRPL11 (L11), and MRPS18B (S18) was performed by kinase enriched fractions eluted from the SCX column at 0.75 and 1 M salt concentrations. The arrow shows the highly phosphorylated subunit of PDH complex, PDHA1. In these assays, kinase enriched fractions were added to reaction mixtures containing 2.5 μ Ci [γ - 32 P] ATP in the absence or presence of 0.3 μ g of recombinant MRPL10, MRPL11, and MRPS18B and incubated for 1 h at 30 $^{\circ}$ C. The assay reactions were separated by SDS-PAGE, fixed, dried, and visualized by phosphor-imaging.

C

Fig. 2. Identification of kinase(s) responsible for phosphorylation of MRPs by LC-MS/MS analysis

A) Equal amounts of 0.75 and 1 M SCX fractions were acetone precipitated and run on a large 10% SDS-PAGE gel to separate the higher molecular weight proteins, where the kinases are most abundant and stained with Coomassie Blue. In-gel tryptic digests of protein bands were analyzed by nano capLC-MS/MS. **B)** Three of the CID MS/MS spectra corresponding to bovine Fyn kinase revealed the presence of this protein in the tryptic digestion of the band shown by an arrow in panel A. Mascot scores for these three doubly charged peptides matching the bovine Fyn kinase were greater than 60. **C)** Primary sequence alignment of bovine SFK members, cSrc (E1BIM8_BOVIN), Yes (A7MB57_BOVIN), and Fyn (A0JNB0). Three peptides detected in the LC-MS/MS analysis are underlined in the alignment showing the uniqueness of two of the peptides for specific identification of Fyn kinase in the fraction. The alignment was created with the CLUSTALW program in Biology Workbench and the results are displayed in BOXSHADE.

Fig. 3. Association of Fyn kinase with MRPs and phosphorylation of MRPs as part of the 55S ribosome *in vitro*

A) Presence of Fyn kinase was detected by immunoblotting analysis in the kinase rich fractions using equal volumes of each fraction. Two of the small and large subunits MRPs, MRPS29 and MRPL47, respectively, were also present in the same fractions. Samples, approximately 30 μ g, were separated on 12% SDS-PAGE gels and membranes were probed with Fyn, MRPS29, and MRPL47 antibodies. **B)** Approximately, 0.2 A_{260} units of purified 55S ribosomes were *in vitro* phosphorylated by [γ - 32 P] ATP in the presence and absence of the 1 M kinase rich fraction using conditions described above. The ribosome in the presence of the kinase buffer served as the control. The arrow indicates the phosphorylation of pyruvate dehydrogenase subunit PDHA1 by PDK as it co-sedimented with the 55S ribosome and 1 M-kinase rich fraction. Phosphorylated samples were run on a 12% SDS-PAGE gel, dried, and visualized by phosphor-imaging.

Fig. 4. Alterations in Fyn kinase expression regulate mitochondrial translation in human cell lines

A) Fyn kinase is constitutively expressed in HepG2 cells at relatively high levels, and its knock-down caused a significant reduction in cells transfected by Fyn siRNA (Fyn) relative to control siRNA (Cont) as detected by the Fyn antibody. Fyn knock-down resulted in major changes in the expression of a mitochondrially encoded subunit of complex IV, COII, at steady-state levels (shown by an arrow). Nuclear-encoded mitochondrial proteins including ATP5A (Complex V), UQCRC2 (Complex III), SDHB (Complex II), and NDUF8 (Complex I) and two MRPs, MRPS29 and MRPL11, were also detected by immunoblotting analyses of whole cell lysates obtained from cells transfected with control and Fyn specific siRNAs.

B) Ectopic expression of Fyn kinase was performed in Hep3B cells with lower or no detectable Fyn expression as shown in cells transfected by the control vector (Cont). Transfection of Hep3B cells with a vector carrying c-Fyn gene resulted in over-expression of Fyn kinase in these cells and stimulation of mitochondrial translation by increasing the expression of COII (shown by an arrow). Detection of nuclear encoded mitochondrial proteins was performed as described in panel A. Blots used in mitochondrial protein detection were stained by Ponceau S (total protein) to ensure equal protein loading. Antibodies used in the analyses were marked on the side of each blot.

Fig. 5. Expression of Fyn kinase is required for oxidative phosphorylation and mitochondrial translation

A) Relative expressions of Fyn kinase, OXPHOS subunits, and mitochondrial ribosomal small and large subunit proteins, MRPS29 and MRPL11, respectively, were compared in HepG2 and Hep3B cell lines detected by the Fyn and OXPHOS antibodies as described in Fig. 4A. **B)** Complex IV activity was determined by measuring the oxidation of cytochrome *c* at 550 nm using equal amounts (~20 µg) of whole cell lysates obtained from HepG2 and Hep3B cells in triplicates as described previously [53]. To calculate the % Relative Change, HepG2 complex IV activity was adjusted to 100%. **C)** *De novo* synthesis of 13 mitochondrially-encoded proteins was evaluated in HepG2 and Hep3B cell lines by pulse labeling of proteins in the presence of [³⁵S]-Methionine and a cytosolic translation inhibitor, emetine. An autoradiograph of the electrophoretic pattern of the *de novo* synthesized mitochondrial translation products is presented. ND1, -2, -3, -4, -4L, -5, and -6 are subunits of Complex I; Cyt *b* is a subunit of Complex III; COI, -II, and -III are subunits of Complex IV; ATP6 and ATP8 are subunits of Complex V. Coomassie Blue staining of the same gel (total protein) was performed to ensure equal protein loading in the gel. Approximately, 30 µg of protein lysates obtained from HepG2 and Hep3B cells were separated on 12% SDS-PAGE prior to immunoblotting and exposure to autoradiography in panels A and C.