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Molecular determinants of cytochrome C oxidase IV mRNA axonal trafficking

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ABSTRACT

In previous studies, we identified a putative 38-nucleotide stem-loop structure (zipcode) in the 3′ untranslated region of the cytochrome c oxidase subunit IV (COXIV) mRNA that was necessary and sufficient for the axonal localization of the message in primary superior cervical ganglion (SCG) neurons. However, little is known about the proteins that interact with the COXIV-zipcode and regulate the axonal trafficking and local translation of the COXIV message. To identify proteins involved in the axonal transport of the COXIV mRNA, we used the biotinylated 38-nucleotide COXIV RNA zipcode as bait in the affinity purification of COXIV zipcode binding proteins. Gel-shift assays of the biotinylated COXIV zipcode indicated that the putative stem-loop structure functions as a nucleation site for the formation of ribonucleoprotein complexes. Mass spectrometric analysis of the COXIV zipcode ribonucleoprotein complex led to the identification of a large number RNA binding proteins, including fused in sarcoma/translated in liposarcoma (FUS/TLS), and Y-box protein 1 (YB-1). Validation experiments, using western analyses, confirmed the presence of the candidate proteins in the COXIV zipcode affinity purified complexes obtained from SCG axons. Immunohistochemical studies show that FUS, and YB-1 are present in SCG axons. Importantly, RNA immunoprecipitation studies show that FUS, and YB-1 interact with endogenous axonal COXIV transcripts. siRNA-mediated downregulation of the candidate proteins FUS and YB-1 expression in the cell-bodies diminishes the levels of COXIV mRNA in the axon, suggesting functional roles for these proteins in the axonal trafficking of COXIV mRNA.

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1. Introduction

It is now evident that one of the mechanisms used by neurons to rapidly respond to developmental or extracellular cues is to temporally and spatially regulate protein expression by asymmetrical mRNA transport and translation (Jung et al., 2012; Gomes et al., 2014). Transcriptome analyses suggest the presence of a highly complex population of RNAs in axons, including messengers that encode proteins that can be organized into functional categories, such as cytoskeletal and scaffolding proteins, translation factors and ribosomal proteins, molecular motors and chaperones, and metabolic enzymes (Willis et al., 2007; Taylor et al., 2009; Zivraj et al., 2010). A major subset of mRNAs that localize to the axon encode mitochondrial proteins (Gumy et al., 2011; Ben-Yaakov et al., 2012; Merianda et al., 2013). These nuclear-encoded mitochondrial proteins are involved in the maintenance of organelle function and promote axonal survival (Gioio et al., 2001). Inhibition of local axonal mRNA translation or the import of mitochondrial proteins synthesized in the axon leads to decreases in mitochondrial membrane potential and the diminution of ATP synthesis in cultured neurons (Hillefors et al., 2007; Natera-Naranjo et al., 2012; Yoon et al., 2012). This finding suggests that the local synthesis and import of nuclear-encoded mitochondrial proteins to axonal mitochondria is essential to support the long-term viability and function of this distal structural domain of the neuron (for review, see Kaplan et al., 2009).

Interestingly, a number of these nuclear-encoded mitochondrial mRNAs code for proteins that play essential roles in oxidative phosphorylation. Studies on two such nuclear-encoded mitochondrial mRNAs, cytochrome C oxidase IV (COXIV), and ATP synthase 9 (ATP5G1) that encode key subunits of oxidative phosphorylation complexes show that local translation of these mRNAs play an important role in the regulation of local axonal energy metabolism, as well as axon function and

Abbreviations: COXIV, cytochrome c oxidase subunit IV; SCG, Superior cervical ganglion; FUS/TLS, Fused in sarcoma/translated in liposarcoma; YB-1, Y-box protein 1; ATP5G1, ATP synthase 9; ROS, Reactive oxygen species; 3′UTR, 3′ untranslated region; ZBP-1, Zipcode binding protein-1; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; RIP, RNA immunoprecipitation.

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growth (Hillefors et al., 2007, Aschrafi et al., 2008, 2012; Natera-Naranjo et al., 2012). For example, disruption of the local translation of axonal COXIV or ATP5G1 mRNAs leads to compromised mitochondrial membrane potential, decreases in ATP levels and generation of reactive oxygen species (ROS) in the axon (Hillefors et al., 2007; Aschrafi et al., 2012; Natera-Naranjo et al., 2012).

Studies investigating cis-acting regulatory elements in COXIV mRNA that mediate the axonal trafficking of the transcript revealed a 38-nucleotide (nt) putative stem-loop structure (zipcode) in the 3′ untranslated region (3′UTR) that was necessary and sufficient to direct the localization of this message to the axon (Aschrafi et al., 2010). Overexpression of the COXIV zipcode in SCG neurons led to decreases in axonal ATP levels, elevated ROS production, as well as defects in axonal growth caused, at least in part, by the reduction in the levels of endogenous axonal COXIV mRNA (Aschrafi et al., 2010; Kar et al., 2014). These decrements in growth and axonal physiology could be partially rescued by decreasing axonal and mitochondrial ROS after application of antioxidants (Aschrafi et al., 2010). Interestingly, overexpression of the COXIV zipcode in select cortical regions of transgenic animals showed increased neuronal ROS and the manifestation of aberrant behavioral phenotypes in young adults (Kar et al., 2014). Taken together, these findings suggest that the axonal transport of nuclear-encoded mitochondrial mRNAs plays an important role in basic neuronal function both in vitro and in vivo.

Selective mRNA transport and local translation of transcripts depends on the interaction of cis-acting regulatory elements in the transcript with RNA binding proteins to form ribonucleoprotein complexes (Doyle and Kiebler, 2012). However, little is known about the proteins that interact with the COXIV zipcode and mediate the trafficking of this transcript to the axon.

To identify the components of the COXIV zipcode ribonucleoprotein complex, we employed a RNA affinity pulldown-coupled mass spectrometry (MS)-based experimental approach. Our results show that the COXIV zipcode binding complex is enriched in RNA-binding proteins, as well as cytoskeletal and mitochondrial proteins. To evaluate the functional significance of the proteins identified by our proteomic approach we selected two candidate proteins: Fused in Sarcoma/Translocated in Sarcoma (FUS/TLS), and Y box binding protein 1 (YB-1) for further analyses. These proteins have been previously shown to be involved in mRNA trafficking and translation (Lagier-Tourenne et al., 2010; Lyabin et al., 2013). Hence, these studies served as “proof of principle” experiments. Results of the RNA affinity purification, western blot analyses and siRNA-mediated knockdown experiments show that the COXIV zipcode indeed interacts with FUS, and YB-1 and that the expression of these proteins can modulate the transport of the COXIV transcript to the axon.

2. Experimental procedures

2.1. Cell cultures

Superior cervical ganglions (SCG) neurons were cultured as described previously (Hillefors et al., 2007). Briefly, SCG were dissected from 3-day-old Harlan Sprague Dawley rat pups and were dissociated using the Miltenyi Biotec gentle MACS Dissociator and the Neuronal Tissue Dissociation Kit according to the manufacturer's protocol. Dissociated primary neurons were plated into the center compartment of a three-compartmented Campenot culture chamber in serum-free Neurobasal medium (Invitrogen) containing nerve growth factor (NGF; 50 ng/ml; Alamone Labs), 20 mM KCl, 20 U/ml penicillin and 20 mg/ml streptomycin (Hyclone) for 2–7 d. The culture medium was changed every 3–4 d. After 2 days-in-vitro (DIV), 5-fluoro-2′-deoxyuridine (FUDR, 50 μM) was added to the medium to inhibit the proliferation of non-neuronal cells. FUDR remained in the medium for the duration of the experiments. Media also contained nerve growth factor at all times. The side compartments, which contained the distal axons used in the experiments, were devoid of neuronal soma and non-neuronal cells, as judged by phase-contrast microscopy. For immunohistochemical studies, dissociated SCG neurons were plated on Nunc® Lab-Tek® II-CC2™ glass chamber slides (Sigma-Aldrich) precoated with collagen and grown for 1-week in culture before being processed for immunostaining.

SHSY5Y cells, a human dopaminergic clonal cell line, were maintained in F12: Dulbecco's modified Eagle's medium (DMEM) 1:1, supplemented with 10% fetal bovine serum, 100 units/ml Penicillin and 0.1 mg/ml streptomycin (Life Technologies). To foster differentiation, approximately 1×10^5 SHSY5Y cells were treated with 10 μM all-trans retinoic acid (RA) and 50 μg/ml NGF for 14 d in Neurobasal media supplemented with B27, GlutaMax, 100 units/ml Penicillin and 0.1 mg/ml streptomycin. Media was replaced every three days.

2.2. Cytosolic and mitochondrial fraction preparation

Cytosolic extracts were prepared from 14-day-old RA-differentiated SHSY5Y cells or 2-week-old compartmentalized SCG cultures using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Life Technologies) according to the manufacturer's instructions. The mitochondrial fractions were isolated from 2-week-old compartmentalized SCG cultures using the Mitochondria Isolation Kit for Cultured Cell according to the manufacturer's protocol. The cell-body and axonal compartments were processed separately. Protein concentration was determined using the Micro BCA Protein Assay Kit (Life Technologies).

2.3. Gel-shift assay

Cytosolic extracts were prepared from differentiated SHSY5Y cells as described earlier. Gel-shift assay was performed by incubating 10 μg of cytosolic extracts in binding buffer (20 mM HEPES, 72 mM potassium chloride, 1.5 mM magnesium chloride, 1.6 mM magnesium acetate, 0.5 mM dithiothreitol [DTT], 4 mM glycerol, 1 mM ATP, 200 units of RNasin; Promega) and 5 pmol of biotin-labeled oligonucleotides for 30 min at room temperature. For competition studies, the binding reactions were performed in the presence of 100-fold molar excess of non-biotinylated oligomer. After incubation, the reaction was fractionated by gel electrophoresis using 4% native polyacrylamide gels and 0.5 × TBE buffer (Biorad). The gel-shift bands were detected using the LightShift Chemiluminescent RNA electrophoretic mobility-shift assay (Life technologies) according to manufacturer's protocol.

2.4. RNA affinity purification

The experimental approach employed in the RNA affinity purification studies was based on a previous study with minor modifications (Kar et al., 2011). Biotinylated RNA oligonucleotides corresponding to the rat COXIV mRNA containing the last 38- and 22-nucleotides of the 3′ untranslated region were synthesized by *IDT technologies*. The 38 nt oligomer is referred to as COXIV full-length zipcode, while the 22 nt oligomer is called the 22 nt deletion oligomer. For the RNA affinity pulldown experiments, 5 pmol of biotinylated RNA oligonucleotide was incubated in a reaction mixture (500 μl) containing 100 μl of differentiated SHSY5Y cytosolic extract in binding buffer (20 mM HEPES/KOH (pH 7.9), 72 mM potassium chloride, 1.5 mM magnesium chloride, 1.625 mM magnesium acetate, 0.5 mM DTT, 4 mM glycerol, 1 mM ATP, 10 μg *E. coli* tRNA), at room temperature for 30 min. After incubation, the RNA-protein mixtures are incubated with 100 μl of magnetic streptavidin beads (Pierce Corporation) preequilibrated in binding buffer for 1 h at room temperature. After three washes with binding buffer, the bead-bound proteins were eluted by boiling for 10 min in elution buffer (125 mM Tris pH 6.8, 2% SDS, 0.02% bromophenol blue, 0.1 M DTT) and size-fractionated by electrophoresis using a 4–12% gradient Bis-Tris SDS polyacrylamide gel (Life Technologies). The affinity purified