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Regulation of axonal trafficking of cytochrome *c* oxidase IV mRNA

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ABSTRACT

Trafficking and local translation of axonal mRNAs play a critical role in the development and function of this neuronal subcellular structural domain. In this report, we studied cytochrome *c* oxidase subunit IV (COXIV) mRNA trafficking into distal axons of primary superior cervical ganglia (SCG) neurons, and provided evidence that axonal trafficking and mitochondrial association of the mRNA are mediated by an element located in a 38 bp-long, hairpin-loop forming region within the 3′UTR of the transcript. Our results also suggest that suppression of local translation of COXIV mRNA results in significant attenuation of axonal elongation. Taken together, the results provide the first evidence for the existence of a *cis*-acting axonal transport element within a nuclear-encoding mitochondrial gene, and demonstrate the importance of the axonal trafficking and local translation of nuclear-encoded mitochondrial mRNAs in axonal growth.

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Introduction

Mitochondria in the axons and presynaptic nerve terminals fulfill distinct functions and have been shown to be closely associated with synapses and tethered to vesicle release sites (Zenisek and Matthews, 2000). To date, little is known about the half-life and biogenesis of synaptically localized mitochondria. Evidence that neuronal mitochondrial biogenesis does occur in the axon at a significant distance from the cell body has been recently provided (Amiri and Hollenbeck, 2008). Over the past few years, it has also become widely accepted that a distinct subset of nuclear-encoding mitochondrial mRNAs are selectively transported to the distal structural/functional domains of the neuron, including the axon and presynaptic nerve terminal (Gioio et al., 2001, 2004; Willis et al., 2007; Taylor et al., 2009). Local proteins synthesized from these mRNAs play a key role, not only for mitochondrial function, but also in the development of the neuron and the function of the axon and nerve terminal. In addition, these studies called attention to the importance of local translation of COXIV mRNA, and its regulation by a brain-specific microRNA, miR-338, that regulates COXIV synthesis locally with distinct consequences on mitochondrial function, such as ATP generation, and axonal function as monitored by neurotransmitter uptake (Aschrafi et al., 2008).

The molecular mechanisms responsible for the transport of COXIV or other nuclear-encoded mitochondrial mRNAs into the axon are unknown. Subcellular mRNA localization and local translation within dendrites and axons are posttranscriptional mechanisms that generally require *cis*-acting sequences for their localization. These gene sequences are usually found in the 3′ untranslated region (3′UTR) of the transcript, and it was suggested that RNA-binding proteins recognize specific secondary structures in the 3′UTR, forming messenger ribonucleoprotein (mRNP) particles that are subsequently transported along microtubules to specific sites (Bassell and Singer, 1997; Kohrmann et al., 1999).

In this work, we used superior cervical ganglia (SCG) neurons cultured in compartmentalized Campenot cultures to examine the axonal transport and translation of COXIV mRNA. Using green fluorescent protein (GFP)-tagged constructs, we identified a 38 bp-long fragment within the COXIV 3′UTR that was required for mitochondrial targeting and axonal localization of the mRNA. Secondary RNA structure analysis of the 3′UTR suggests that these *cis*-acting regulating sequences are situated in a hairpin-loop forming region. In addition, our investigations identified local COXIV synthesis as an important determinant of axonal growth, as overexpression or knockdown of axonal COXIV levels significantly increased or attenuated neurite elongation, respectively.

Results

The COXIV 3′UTR is sufficient for axonal mRNA transport

Most of the *cis*-acting sequences that have been identified as being involved in dendritic or axonal localization are situated in the 3′UTR of the mRNA (Martin and Zukin, 2006). Initial comparative sequence analysis revealed that the 3′UTR of the mammalian COXIV gene is highly conserved (Fig. 1A). In addition, an RNA secondary structure

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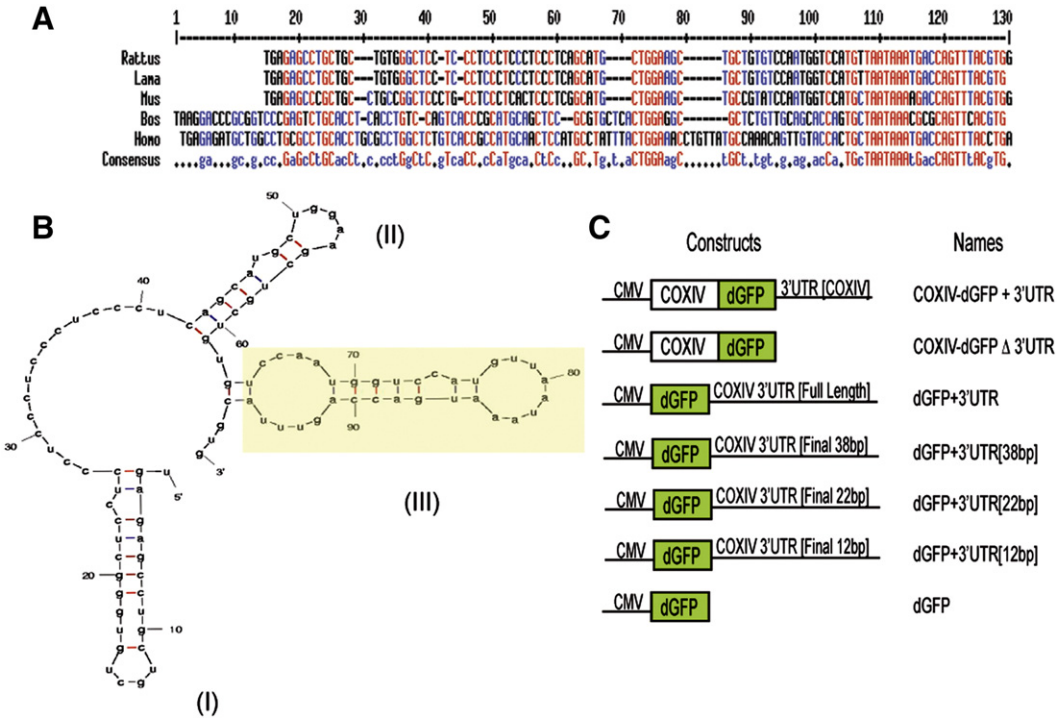

Fig. 1. The 3′UTR of mammalian COXIV mRNA is highly conserved. (A) Sequence alignment of the 3′UTR of COXIV mRNAs from five mammalian species shows a high degree of sequence conservation. The red and the blue shading represent nucleotides conserved among all five and in four out of the five species, respectively. Each 3′UTR sequence begins with the stop codon. (B) Structure of the COXIV 3′UTR, as determined by secondary structure prediction analysis (Mfold). Yellow highlighted hairpin-loop segment (III) facilitates mRNA transport into distal axons. (C) Overview of constructs used in this study. Plasmid constructs consisting of the COXIV ORF followed by the destabilized GFP (dGFP) cDNA that contain the COXIV 3′UTR [+3′UTR] or the SV40 3′UTR [Δ3′UTR], are diagrammed. In addition, schematic representation of reporter gene plasmids carrying the dGFP cDNA followed by either the full-length 3′UTR of rat COXIV, the final 38 bp, 22 bp or 12 bp of the COXIV 3′UTR, respectively, is shown. Expression of all fusion gene products was driven by the human cytomegalovirus promoter (CMV).

prediction analysis using Mfold (Zuker, 2003) indicated that this 3′UTR consisted of three well-conserved stem–loop structures, that might have functional significance (Fig. 1B). Previous investigation of the COXIV 3′UTR confirmed that the target site for the brain-specific miR-338 was positioned on the second hairpin-loop structure, in an exposed position facilitating microRNA accessibility and subsequent regulation of COXIV expression (Aschrafi et al., 2008).

Our previous studies also suggested that the nuclear-encoded COXIV mRNA is localized constitutively in relatively high abundance in the distal axons and presynaptic terminals of sympathetic neurons (Hillefors et al., 2007; Aschrafi et al., 2008). To determine whether the 3′UTR of COXIV mRNA contained functional transport signals for directing mRNAs into distal axons, we employed an experimental strategy that encompassed the transfection of chimeric gene expression plasmids into primary SCG neurons. These chimeric gene constructs consisted of the COXIV ORF followed by a destabilized GFP cDNA (dGFP; with a known protein half-life of approximately 2 h) that contained the COXIV 3′UTR [COXIV-dGFP + 3′UTR], or the SV40 3′UTR [COXIV-dGFPΔ3′UTR] (Fig. 1C). These vectors expressing chimeric reporter mRNAs were introduced into SCG neurons by transfecting the soma and proximal axons located in the center compartment of Campenot chambers (see scheme Fig. 2A). One to two days after transfection, we used GFP-specific primers and qRT-PCR to quantify COXIV-dGFP mRNA levels in the distal axons of SCG neurons that were located in the lateral compartments of the Campenot cultures. It has been previously shown that somatically introduced GFP transcripts remain restricted to the somata of sympathetic neurons (Muslimov et al., 1997). As shown in Fig. 2A, COXIV-dGFP mRNAs lacking the 3′UTR of COXIV were detected at similar levels as the dGFP mRNAs in total RNA prepared from distal axons, while levels of the chimeric mRNAs carrying the 3′UTR of COXIV were present at significantly higher levels in the axonal compartment. This observation suggested that the COXIV 3′UTR was both necessary and sufficient for targeting COXIV mRNAs to the distal axons. Following the transfection protocols described above, we also tested whether the COXIV mRNAs targeted to the distal axons were translated locally. In previous studies, the local translation of functionally important mRNAs in distal parts of neurons has been demonstrated utilizing destabilized GFP reporter constructs (Macchi et al., 2003). Fluorescence microscopy was employed to visualize dGFP expression in the distal axons of SCG neurons. The experiments revealed that the 3′UTR of COXIV significantly increased axonal expression of the COXIV-dGFP chimera, as compared to COXIV-dGFP lacking the COXIV 3′UTR, and dGFP, respectively (Fig. 2B). In addition, a significant amount of the COXIV-dGFP fluorescence was localized to small, punctuate structures. Next, mitochondrial association of the fusion proteins was assessed using co-localization studies in axons expressing the chimeric mRNAs followed by axonal staining with MitoTracker Red, a mitochondrion-specific fluorescent dye (Fig. 2B). This analysis revealed that the N-terminally located COXIV amino acid sequence was able to target the COXIV-dGFP fusion protein to the mitochondria, as shown in yellow in the merged images in Fig. 2B. This finding indicates that the translated COXIV-dGFP is targeted to the mitochondria.

The 3′UTR of COXIV mRNA contains an axonal targeting element

To identify the *cis*-acting axonal targeting sequences in the 3′UTR of the rat COXIV mRNA, we created vectors expressing chimeric reporter mRNAs. In the control vector, a transcript encoding dGFP is synthesized under the control of the CMV promoter and contains a SV40 3′UTR. It has been shown previously that the mRNA derived from such a construct is restricted to cell bodies of primary neurons (Goetze et al., 2003). To investigate whether discrete regions of the COXIV 3′UTR are capable of facilitating axonal localization of the dGFP