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The mitochondrial fatty acid synthesis (mtFASII) pathway is capable of mediating nuclear-mitochondrial cross talk through the PPAR system of transcriptional activation

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ABSTRACT

Mammalian cells contain two fatty acid synthesis pathways, the cytosolic FASI pathway, and the mitochondrial FASII pathway. The selection behind the conservation of the mitochondrial pathway is not completely understood, given the presence of the cytosolic FAS pathway. In this study, we show through heterologous gene reporter systems and PCR-based arrays that overexpression of MECR, the last step in the mtFASII pathway, causes modulation of gene expression through the PPAR pathway. Electromobility shift assays (EMSAs) demonstrate that overexpression of MECR causes increased binding of PPARs to DNA, while cell fractionation and imaging studies show that MECR remains localized to the mitochondria. Interestingly, knock down of the mtFASII pathway lessens the effect of MECR on this transcriptional modulation. Our data are most consistent with MECR-mediated transcriptional activation through products of the mtFASII pathway, although we cannot rule out MECR acting as a coactivator. Further investigation into the physiological relevance of this communication will be necessary to better understand some of the phenotypic consequences of deficits in this pathway observed in animal models and human disease.

1. Introduction

Mammalian cells contain at least two fatty acid synthesis (FAS) pathways, the cytosolic FASI pathway, and the mitochondrial FASII pathway. The cytosolic pathway is catalyzed by one multifunctional protein, fatty acid synthase, that carries out all of the steps necessary for fatty acid synthesis [1]. The mitochondrial fatty acid synthesis pathway (mtFASII) is reminiscent of prokaryotic FAS systems in that it consists of a separate protein performing each catalytic step [2,3]. The selection behind the conservation of the mitochondrial pathway is not completely understood, given the presence of the cytosolic FAS pathway. While the main function of the ancestral type II system in bacteria is the synthesis of glycerophospholipids, the pathway also creates intermediates that are diverted to synthesize other molecules, such as lipopolysaccharides, vitamins such as the protein-bound coenzymes biotin and lipoic acid, and the acylated homoserine lactones involved in density-dependent signaling [2,3].

In eukaryotic cells, the fate of mtFASII pathway products is less certain. The mtFASII pathways of *Neurospora* [4,5], plants [6,7], and mammals [8] have all been demonstrated to synthesize saturated fatty acids from malonate. Because most of the lipids in mitochondria are imported from the cytoplasm where they were synthesized by the FASI pathway [9,10], *de novo* synthesis of phospholipids from these fatty acids is unlikely. Experiments in fungi, however, suggest that these mtFASII products may be important in phospholipid side chain remodeling. Deletion of *acp-1*, the gene for the mtFASII acyl carrier protein in *Neurospora*, results in a 4-fold increase in mitochondrial lysophospholipids [11]. Deletion of mtFASII pathway genes in *Saccharomyces cerevisiae* results in a more than 50% reduction in cardiolipin levels [12,13], and loss of 90–95% of the normal level of lipoic acid [12].

Some understanding of the role of mammalian mtFASII has come from knocking down expression of pathway components in cultured cells and mice. ACP is a small (8 kD) mitochondrial protein essential to mtFASII, acting as a carrier for the fatty acids as they are elongated. ACP has also been identified as a component of respiratory chain complex I in *Neurospora crassa* and mammals [14–16]. siRNA-medicated knock down of the gene for ACP (*NDUFAB1*) in HeLa cells led to decrease in lipoylated proteins, slower cell growth, reduction in the specific activity of complex I of the electron transport chain, and loss of mitochondrial membrane

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potential [17]. Knock down of *Mcat*, the gene for malonyl CoA-acyl carrier protein transacylase that converts malonyl-CoA to malonyl-ACP in the mtFASII pathway, results in a dramatic phenotype in mouse, including shortened life span, baldness, weight loss despite food intake, loss of white fat, and hypothermia [18] emphasizing the important physiological role of this pathway in mammalian systems.

Upregulation of another mtFASII pathway enzyme, mitochondrial trans-2-enoyl-CoA reductase (MECR), in transgenic mouse heart resulted in cardiac dysfunction and dysmorphic mitochondria [19]. MECR, which catalyzes the last step in the mtFASII pathway [20], was originally identified as interacting with peroxisome proliferator-activated receptor α (PPARα) in the two-hybrid system [21]. The transcriptional activity of the PPARs is modulated by ligands such as the hypolipidemic fibrate drugs that cause peroxisome proliferation, and naturally occurring ligands such as fatty acids, eicosanoids, and phospholipids. These ligands affect the binding of proteins (coactivators and corepressors) that modulate the transcriptional activity of the PPARs [22].

Although MECR also interacts with several other nuclear hormone receptors, evidence of its ability to affect transcription has not been demonstrated. In this study, we show that upregulation and downregulation of members of the mtFASII pathway cause modulation of PPARdriven expression.

2. Materials and methods

2.1. Plasmid construction

To create the MECR overexpression plasmid, the entire open reading frame of *Mecr* was amplified from mouse cDNA using primers 5′-CCAGATCTGCCGCCACCATGGTGGTCAGCCAGCGAGTG-3′ and 5-TGGAGAGATCTCATGGTGAGAATCTGCTTCG-3. The resulting PCR product was cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen) to create pMecr-TA. A FLAG epitope tag was created at the C terminus of *Mecr* by annealing complementary oligonucleotides 5′-GATCCACCATGGATTACAAGGATGACGTACGATAAGA-3′ and 5′-GATCTCTTATCGTCGTCATCCTTGTAATCCATGGTG-3′ and ligating the resulting product into the pMecr-TA vector that had been digested with BglII, creating pMecr-flagTA. The region encoding MECR-flag was then removed by digestion with EcoRI and BglII, and cloned into pSG5 (Agilent Technologies, Inc.), creating a plasmid expressing *Mecr* under the control of the SV40 promoter.

To create the reporter plasmid containing the *Mecr* promoter controlling luciferase expression, the region 700 bp upstream of the start codon of *Mecr* was amplified from mouse genomic DNA and cloned into the pCR2.1 vector using the TA cloning system (Invitrogen). The *Mecr* promoter region was then excised from pCR2.1 using EcoRI, and ligated into EcoRI site of pGL2-basic (Promega) upstream of the luciferase gene.

Mitochondrially targeted dsRED (m-dsRED) was a gift from J. Nunnari at University of California, Davis [23].

MECR–GFP was constructed by amplifying the entire open reading frame of *Mecr* from mouse cDNA using primers listed above, and ligation of the PCR product cut with BglII into the BglII site of pEGFP-N3 (gift from the D. Piston laboratory, Vanderbilt University).

2.2. Co-activation assays

To test if MECR has an effect on PPAR-driven transcription, a luciferase reporter-based transcriptional activation assay was used. The promoter of the carnitine palmitoyltransferase Ib gene (*Cpt1B*) [24], or acyl coA oxidase (*Aco1*) gene [25], both of which contain PPAR response elements (PPREs), were independently used to drive luciferase reporter gene expression. The reporter plasmid (CPT-luc or ACO-luc) was transiently co-transfected into HeLa cells using FuGene HD (Roche) with plasmids expressing peroxisome proliferator-activated receptor alpha (Pparα) [26] or gamma (Pparγ) and retinoid X receptor alpha (Rxrα) [26] transcription factors. The contribution of MECR was assessed by cotransfection of a plasmid driving overexpression of mouse *Mecr* under control of the SV40 promoter.

2.3. Cell fractionation/western blot

HeLa cells were transfected with either MECR or control (pSG5) plasmid and PPARα and RXRα plasmids. Cells were harvested and fractionated using the standard cell fractionation kit from Mitosciences. Antibodies specific to each cell fraction where used for Western immunoblots: GAPDH (Santa Cruz Biotechnology sc-20357) for cytosol, Lamin A/C (Cell Signaling Technology 4C11) for nuclear, and PDH (Abcam/Mitosciences MSp06) for mitochondrial. Polyclonal MECR antibodies were created by immunizing rabbits with mouse MECR-specific synthetic peptide (CSEVPLQGYQQALEASMKPF) conjugated to keyhole limpet hemocyanin (KLH) (Proteintech). MCAT antibody (sc-390858) is from Santa Cruz Biotechnology.

2.4. Confocal fluorescent microscopy

Confocal microscopy of transfected HeLa cells was conducted on a Zeiss LSM 510 with a 65X oil-immersion objective. M-dsRED and MECR–GFP transfected cells were imaged sequentially following excitation by lasers emitting at 543 and 488 nm, respectively.

2.5. Electrophoretic mobility shift assays

Evaluation of binding of transcription factors to DNA was performed using the PPAR EMSA kit from Signosis according to manufacturer instructions. Nuclear extracts were isolated using the nuclear extraction kit from Cayman Chemicals. Labeled probe was incubated with nuclear extracts from HeLa cells transfected with MECR, PPARα, RXRα, or control expression vectors. Flag M2 monoclonal antibodies for supershift experiments were obtained from Sigma. PPAR antibodies for supershift experiments were obtained from Santa Cruz Biotechnology.

2.6. siRNA medicated RNA knock down

Knock down of the mtFASII pathway was achieved using Qiagen Flexitube siRNAs specific for the gene for ACP (*NDUFAB1*) or MCAT (*MCAT*). HeLa cells were transfected with siRNA using HiPerfect Transfection Reagent (Qiagen). Knock down efficiency was measured after 96 h using real time quantitative RT-PCR or by Western immunoblot for MCAT.

2.7. Real time quantitative RT-PCR

Total RNA was isolated using TRIzol Reagent (Life Technologies) according to manufacturer's protocols. First-strand cDNA was created from total RNA using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies). Quantitative RT-PCR was performed using TaqMan Expression Assays (Life Technologies) on the ABI 7900 platform according to manufacturer's protocols.