A Novel Cytosolic Isoform of Mitochondrial Trans-2-Enoyl-CoA Reductase Enhances Peroxisome Proliferator-Activated Receptor α Activity

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Background: Mitochondrial trans-2-enoyl-CoA reductase (MECR) is involved in mitochondrial synthesis of fatty acids and is highly expressed in mitochondria. MECR is also known as nuclear receptor binding factor-1, which was originally reported with yeast two-hybrid screening as a binding protein of the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα). However, MECR and PPARα are localized at different compartment, mitochondria, and the nucleus, respectively. Therefore, the presence of a cytosolic or nuclear isoform of MECR is necessary for functional interaction between MECR and PPARα.

Methods: To identify the expression pattern of MECR and the cytosolic form of MECR (cMECR), we performed reverse transcription polymerase chain reaction (RT-PCR) with various tissue samples from Sprague-Dawley rats. To confirm the interaction between cMECR and PPARα, we performed several binding assays such as yeast two-hybrid, coimmunoprecipitation, and bimolecular fluorescence complementation. To observe subcellular localization of these proteins, immunocytochemistry was performed. A luciferase assay was used to measure PPARα activity.

Results: We provide evidence of an alternatively spliced variant of the rat MECR gene that yields cMECR. The cMECR lacks the N-terminal 76 amino acids of MECR and shows uniform distribution in the cytoplasm and nucleus of HeLa cells. cMECR directly bound PPARα in the nucleus and increased PPARα-dependent luciferase activity in HeLa cells.

Conclusion: We found the cytosolic form of MECR (cMECR) was expressed in the cytosolic and/or nuclear region, directly binds with PPARα, and enhances PPARα activity.

Keywords: Trans-2-enoyl-CoA reductase (NADPH); Cytosolic mitochondrial trans-2-enoyl-CoA reductase; PPAR alpha; Alternative splicing; Mitochondrial targeting sequences

INTRODUCTION

Mammalian mitochondria perform nicotinamide adenine di-nucleotide phosphate (NADPH)-dependent de novo fatty acid synthesis (FAS) [1]. This mitochondrial FAS pathway resembles the well-understood bacterial FAS II pathway [2]. Mito-
chondrial trans-2-enoyl-CoA reductase (MECR) is a component of the mitochondrial FAS II pathway and catalyzes the fatty acid elongation cycle in the last step, which is the NADPH-dependent reduction of the enoyl-acyl carrier protein substrate [3-5]. MECR is primarily localized in mitochondria [6-8], and the N-terminal amino acids of this protein are important for its mitochondrial localization [9]. MECR is also known as nuclear receptor binding factor-1 (NRBF-1) because, using yeast two-hybrid screening, it was identified as a binding protein of the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα) [10]. However, the biological meaning of the interaction between MECR and PPARα has not been determined.

PPARα is a ligand-activated transcription factor that is one of three different PPAR subtypes: PPARα, PPARβ/δ, and PPARγ. The PPARs play important roles in nutrient homeostasis [11-13] and are localized in the nucleus. Although MECR was previously reported as a binding protein of PPARα [10], interaction between MECR and PPARα seems not to occur in mammalian cells due to their different subcellular localizations, mitochondria and the nucleus, respectively. Therefore, the presence of a cytosolic or nuclear isoform of MECR is necessary for functional interaction between MECR and PPARα in the nuclei of cells.

Here, we analyzed the expression pattern of MECR in several rat tissues and found a novel splice variant of MECR in which an additional exon was inserted between exon 1 and exon 2. The protein generated from this splicing variant has an N-terminal region that does not contain the mitochondrial targeting signal peptide and thus is not localized in mitochondria. Moreover, this MECR variant bound PPARα in the nucleus and enhanced PPARα transcriptional activity. Based on these results, we propose that this novel variant of MECR, cytosolic MECR (cMECR), plays a role in intracellular signal pathways as an interacting partner of PPARα.

METHODS

RNA extraction and reverse transcription polymerase chain reaction

Total RNA was extracted from tissues of male Sprague-Dawley rats (14 weeks old) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Total RNA (1 μg) from each sample was reverse transcribed using random primers (50 pmol), SuperScript III Reverse Transcriptase (Invitrogen), and dNTPs (1 mM) at 42°C for 1 hour. The forward primer (5’-ATGTTGGTCAGCCGGC-186GACT-3’) and reverse primer (5’-TCACATAGTGAGAATCTGCT-3’) were designed to amplify full-length MECR. Additional primers were used for specific detection of MECR and cMECR cDNA: forward primer (5’-GTCGTGAAGC GGCAATGTTG-3’) and reverse primer (5’-TGAGCTCCAGGTTCCTCAG-3’). cMECR and PPARα fragments were amplified under the following cycle conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. This cycle was repeated 35 times. Glyceraldehyde 3-phosphate dehydrogenase fragments were also amplified under the same conditions except that 25 cycles were run. Polymerase chain reaction (PCR) products were analyzed with 2.0% agarose gel electrophoresis, purified, and ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant plasmids were sequenced.

Construction of plasmids and antibodies

The rat full-length MECR (GenBank accession no. AB015724), cytosolic variant of MECR, and PPARα (GenBank accession no. NM_001001928) were cloned using the Gateway Cloning System (Invitrogen) as previously described [14]. After amplification of the three genes, PCR products were cloned into the pDONR207 vector and then subcloned into self-constructed destination vectors such as pDs_XB-HA, pDs_XB-Flag, pDs_XB-enhanced green fluorescent protein (EGFP), and pDs_XB-mCherry expression vectors. Anti-Flag (F6531) antibody was purchased from Sigma (St. Louis, MO, USA). Anti-EGFP (B-2) and antihemagglutinin (anti-HA) (F-7) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and transfection

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin in a humid atmosphere containing 5% CO₂ at 37°C. When needed, cells were seeded onto cover slips for imaging analysis or 60-mm dishes for preparation of lysates for Western blot analysis. Transfected cells were cultured for an additional 24 hours in growth medium and then used for further analysis.

Coimmunoprecipitation and Western blot analysis

Transfected cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1 mM phenylmethanesulfo-
nyl fluoride, and 1% NP-40) containing a protease inhibitor cocktail (Sigma). Whole-cell lysates were incubated on ice for 30 minutes and then cleared at 13,000 rpm for 20 minutes at 4°C. The immune complexes were incubated for 1 hour at 4°C with gentle rotation with 20 μL protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) that had been prewashed and suspended in 100 mL cold lysis buffer. The proteins were separated with 10% SDS-PAGE and blotted onto polyvinylidene fluoride membranes. The blots were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (20 mM Tris-buffered saline and 0.05% Tween 20, pH 7.5) at room temperature for 20 minutes and incubated overnight at 4°C with anti-GFP antibody (1:1,000), anti-HA antibody (1:1,000), or anti-FLAG antibody (1:1,000). Blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000), followed by washing and detection of immunoreactivity with enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

**Imaging analysis**

Subcellular distribution of MECR, cMECR, and PPARα were confirmed according to the EGFP and mCherry fluorescence detection method with a confocal microscope (Olympus Fluoview FV1000, Olympus, Tokyo, Japan). For imaging analysis, cells were plated onto glass cover slips. After transfection, the cells were grown for 24 hours and treated with the mitochondrion-selective fluorescent dye mito-tracker red CMXRos (Molecular Probes Europe BV, Leiden, Netherlands) according to the manufacturer’s instructions.

**Yeast two-hybrid assay**

cMECR was cloned into pGADT7 encoding the activation domain, and PPARα was ligated into pGBK7 encoding the GAL4 DNA binding domain. To evaluate the protein-protein interaction between cMECR and PPARα, both pGAD-cMECR and pGBK-PPARα were cotransformed into the yeast strain AH109. This strain is unable to synthesize histidine. However, interaction between cMECR and PPARα enables the yeast to make the His3 enzyme, thereby permitting histidine biosynthesis and growth on His minimal medium.

**Bimolecular fluorescence complementation assays**

C- and N-terminal venus plasmids were purchased from Addgene and modified to contain cMECR or PPARα with subcloning. Cells were transfected with the lipofectamine 2000 reagent (Invitrogen) with venus plasmids containing cMECR and PPARα. After 24 hours, cells were imaged with confocal microscopy as described above.

**Luciferase assay**

HeLa cells were plated at a density of 1.0 to 1.5×10⁵ cells per well onto six-well dishes. Twenty-four hours later, cells were cotransfected with 1 μg of PPAR responsive element (PPRE)-3x-TK-Luc, the firefly luciferase reporter gene containing PPRE; 0.2 μg of the PPARα expression vector; and either the MECR expression vector or cMECR expression vector. Transfections were accomplished with lipofectamine 2,000 reagent according to the manufacturer’s recommendations. The PPRE-3x-TK-Luc plasmid includes three PPREs upstream of an inducible thymidine kinase promoter controlling transcription of the firefly luciferase gene. Cells were harvested 24 hours after the start of exposure to vehicle or inducing agent. Luciferase assays were performed using the Luciferase Assay System (Promega) according to the manufacturer’s protocol.

**RESULTS**

**Identification of a novel alternative transcript variant of MECR**

During reverse transcription (RT)-PCR analysis of MECR expression in the rat brain, two different MECR transcripts were detected. One was the expected MECR mRNA (210 bp), which corresponds to the known MECR mRNA (GenBank accession no. AB015724). The other was a longer 306-bp PCR fragment (Fig. 1C). Because PCR was carried out with specific primers corresponding to exons 1 and 2 of rat MECR cDNA, alternative splicing was proposed to account for the presence of this fragment. Fig. 1A shows the 10 exons of rat MECR cDNA. The novel MECR variant has an additional 96 nucleotides between exon 1 and exon 2. Basic local alignment search tool analysis of the aligned sequences of rat genomic DNA (chromosome 5 genomic contig NW_047784) and cDNA of this MECR variant showed that the flanking nucleotides of convergent points between exon 1 and exon 2 followed the GT/AG rule of alternative splicing (Supplementary Fig. 1A). This addition of 96 nucleotides generated a frame-shifted MECR, and the N-terminal 76 amino acids were deleted by use of another start codon in exon 3 (Fig. 1B). Because this splice variant lacked the N-terminal mitochondrial targeting sequences, we
Fig. 1. Identification of a novel variant of mitochondrial trans-2-enoyl-CoA reductase (MECR; cytosolic form of MECR [cMECR]) cDNA. (A) The genomic structure of MECR and cMECR and their coding regions are shown. Inverted triangles indicate the start and stop codons. The dotted-line box indicates the inserted nucleotide sequence. (B) Alignment of the MECR and cMECR sequences. The positions of the upper and lower primers are marked with arrows. (C) Expression patterns of MECR and cMECR in various tissues. The upper polymerase chain reaction band is cMECR, and the lower band is MECR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Pa, pancreas; Sp, spleen; Th, thymus. *Each start codon.
named it cMECR.

Next, to compare expression of cMECR and MECR, RT-PCR analyses were done using RNA samples from eight different tissues with a specific primer set as shown in Fig. 1B. As shown in Fig. 1C, cMECR (306 bp) and MECR (210 bp) were ubiquitously expressed in most tissues examined, although the expression level of cMECR was lower than that of MECR. Furthermore, we found amino acid sequences of human MECR isoform 2 (GenBank accession no. NP001019903.1) were well matched with that of rat cMECR (Supplementary Fig. 1B). These data raise the possibility that cMECR can be produced in other species.

cMECR localized in the cytoplasm and nucleus of HeLa cells

To visualize the intracellular distribution of MECR in HEK cells, we generated two constructs containing EGFP-tagged full-length rat MECR. As shown in Supplementary Fig. 2, when EGFP was tagged to the C-terminal end of MECR (MECR-EGFP), this protein was distinctly expressed in mitochondria, as previously reported [3]. However, the N-terminal EGFP-tagged MECR (EGFP-MECR) was diffusely expressed in the cytoplasm of HEK cells. N-terminal tagging of the protein with EGFP might have disrupted the proper mitochondrial localization of MECR, perhaps because the N-terminal mitochondrial targeting sequence of MECR was masked by the EGFP. These results suggest that N-terminal mitochondrial targeting sequences are critical for mitochondrial localization of MECR proteins.

Because cMECR lacks the N-terminal mitochondrial targeting sequences, the intracellular distribution of this protein was unclear. The intracellular distribution of MECR and cMECR was thus compared by tagging both cDNAs with EGFP at their C-terminal ends and then transiently expressing them in HeLa cells (Fig. 2). MECR-EGFP showed a well-defined punctate pattern that clearly colocalized with Mito tracker, a marker of mitochondria (Fig. 2B). However, cMECR-EGFP was expressed in cytosolic and nuclear regions and did not colocalize with Mito tracker (Fig. 2C).
cMECR directly interacts with PPARα

MECR, also known as NRBF-1, was previously identified with yeast two-hybrid screening as a PPARα-interacting protein [10], although explaining how mitochondrial-targeted MECR interacted with the nuclear hormone receptor PPARα was difficult. In contrast to MECR, cMECR was localized in the cytoplasm and nucleus of HeLa cells, and its amino acid sequence is exactly the same as that of MECR, except for the N-terminal 76 amino acids that compose the mitochondrial targeting sequence. Thus, cMECR, rather than mitochondrial MECR, could interact with PPARα in the nucleus. Indeed, the yeast two-hybrid assay showed a direct interaction between cMECR and PPARα (Fig. 3A). Immunoprecipitation and colocalization analyses also showed that cMECR interacted with PPARα in the nucleus (Fig. 3B, C). Taken together, these data strongly indicate that cMECR, but not MECR, is a bona fide binding partner of PPARα.

cMECR binds with PPARα in the nucleus and potentiates PPARα activity

To more clearly investigate whether the interaction between cMECR and PPARα can occur in the nucleus of HeLa cells, we performed a bimolecular fluorescence complementation (BiFC) experiment. The BiFC assay provides a direct approach for the visualization of molecular interactions in living cells.
Fig. 3. Cytosolic form of mitochondrial trans-2-enoyl-CoA reductase (cMECR) directly interacts with peroxisome proliferator-activated receptor α (PPARα) in vitro. (A) Yeast two-hybrid assay between cMECR and PPARα: on a Trp−/Leu−/His− plate, colonies indicate an interaction between the two genes. (B) Coimmunoprecipitation assays with cMECR and PPARα: cMECR-FLAG was cotransfected with hemagglutinin (HA)-PPARα in HeLa cells. The lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-HA antibody. (C) Colocalization of cMECR and PPARα: HeLa cells were transiently transfected with cMECR-enhanced green fluorescent protein (EGFP; green) and mCherry-PPARα (red). Scale bar=20 μm.

Supplementary Fig. 3. Localization of mitochondrial trans-2-enoyl-CoA reductase (MECR) and peroxisome proliferator-activated receptor α (PPARα): wild-type MECR is localized in cytosolic regions, whereas PPARα is localized in nuclear regions. HeLa cells were transiently transfected with MECR-enhanced green fluorescent protein (EGFP; green) and mCherry-PPARα (red). Scale bar=20 μm.
MECR has been reported to be a mitochondrial protein [7] that catalyzes NADPH-dependent reduction of trans-2-enoyl thiosteres to the corresponding saturated acyl thiosteres [5]. However, NRBF-1 (also known as MECR) was originally identified as a binding protein of the nuclear receptor PPARα [10]. In that paper, the authors did not perform any assessment of endogenous binding in cells or report the biological meaning of this protein–protein interaction, although NRBF-1 binds with PPARα in the yeast two-hybrid system.

Here, we report a cMECR that is generated by alternative splicing. cMECR is an N-terminal-76-amino-acid truncated...
isoform of MECR. Because mitochondrial targeting signal sequences are contained between amino acids 1 and 53 of MECR [8], cMECR lacks this mitochondrial targeting signal sequence and was preferentially localized to cytosolic and nuclear regions and was not present in mitochondria (Fig. 2C). In addition, our data show that N-terminal EGFP-tagged MECR also lost its proper intracellular localization (Supplementary Fig. 2). Therefore, the N-terminal region of MECR is critical for mitochondrial localization of the MECR protein.

Different intracellular distribution of cMECR strongly implies that its biological functions differ from the functions of MECR. In addition, cMECR mRNA was ubiquitously expressed in eight tested tissues (Fig. 1C), implying that cMECR could perform common essential functions in the various tissues. Although further studies should be performed to elucidate the function(s) of cMECR, we present its first function in this study. Various nuclear receptors activate transcription of their target genes through interactions with coactivators or corepressors [17,18]. Like other coactivators, cMECR directly bound PPARα in the nucleus and potentiated PPARα activity (Fig. 4).

Generally, PPARα is found in tissues where fatty acid metabolism is important and regulates genes involved in lipid and lipoprotein metabolism as well as glucose homeostasis [19-21]. Because cMECR potentiates PPARα activity in HeLa cells, cMECR could be involved in these biological phenomena. An earlier study used the yeast two-hybrid assay and demonstrated that MECR (NRBF-1) also interacts with various nuclear hormone receptors such as TRβ, RARα, RXRα, and HNF-4 [10]. Because our data strongly support the idea that cMECR is a bona fide binding partner of PPARα and its amino acid sequence is the same as that of MECR except that it lacks the N-terminal 76 amino acids, cMECR might also bind with other nuclear hormone receptors. This possibility should be tested in future studies.

In summary, our results suggest that cMECR, a novel alternatively spliced variant of MECR, directly interacts with PPARα in the nucleus and could be a positive regulator in the physiological regulation of PPARα.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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