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The Adaptor-Related Protein Complex 2, Alpha 2 Subunit (AP2 α 2) Gene is a Peroxisome Proliferator-Activated Receptor Cardiac Target Gene

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Abstract

A peroxisome proliferator-activated receptor (PPAR) response element (RE) in the promoter region of the adaptor-related protein complex 2, alpha 2 subunit (AP2 α 2) of mouse heart has been identified. The steroid hormone nuclear PPARs and the retinoid X receptors (RXRs) are important transcriptional factors that regulate gene expression, cell differentiation and lipid metabolism. They form homo- (RXR) and hetero- (PPAR-RXR) dimers that bind DNA at various REs. The AP2 α 2 gene is part of complex and process that transports lipids and proteins from the plasma membrane to the endosomal system. A PPAR activator (Wy14643) and DMSO (vehicle) was introduced into control and 8337T thyroid hormone receptor (TR β 1) transgenic mice. Heart tissue was extracted and AP2 α 2 gene expression was compared using Affymetrix expression arrays and qRT PCR among four groups [control, control with Wy14643, 8337T TR β 1 and 8337T TR β 1 with Wy14643]. The gene expression of AP2 α 2 in the Wy14643 control and transgenic mouse groups was significantly up regulated over the vehicle mouse groups in both the array ($p<0.01$) and qRT PCR ($p<0.01$) studies. Duplex oligo DNAs containing the PPAR/RXR motif (AGGTCA/TCCAGT) from the AP2 α 2 promoter were used in EMSA to verify binding of the PPAR and RXR receptors to their REs. pGL4.0 [Luc] constructs of the AP2 α 2 promoter with and without the PPAR/RXR motifs were co-transfected with mouse PPAR α , β or γ 1 into HepG2 cells and used in luciferase assays to verify gene activation. In conclusion our study revealed that PPAR α regulates the mouse cardiac AP2 α 2 gene in both the control and transgenic mouse.

Keywords

AP2 α 2; PPARs; PPREs; Cardiac function; Gene regulation

1 Introduction

The heterotetrameric AP2 protein complex is involved in the formation of clathrin-coated pits and functions as an adaptor by linking receptors directly to the clathrin lattice [16, 28, 31]. AP2 is a stable complex comprised of four non-identical polypeptide chains: the α 2-subunit (~100 kDa), the β 2-subunit (100 kDa), the μ 2-subunit (50 kDa) and the σ 2-subunit (17 kDa) [34]. One class of transport vesicles are the clathrin-coated vesicles (CCVs), so called because the major component of the coat is clathrin [27]. Clathrin-mediated endocytosis (CME) is the main process by which receptors and other integral membrane proteins and lipids are removed from the plasma membrane and delivered into the endosomal system. CME plays a central role in regulating plasma membrane protein composition by defining the interaction of a cell with its surroundings, by transduction of mitogenic signals and uptake of nutrients across the cell boundary, by establishing cellular identity to the extracellular environment including interaction with the immune system, and by maintaining cellular homeostasis [9]. The protein adaptor in CCVs mediating CME is the AP2 complex [29], which binds to both protein cargo and plasma membrane phospholipid head groups [11, 24] as well as to clathrin. The α subunit (α -adaptin) of AP2 binds clathrin and has been implicated in interacting with dynamin [12, 37], a GTPase involved in the budding of CCVs [17]. This subunit also interacts with *Eps15*, a protein that both binds and acts as a substrate for the epidermal growth factor receptor tyrosine kinase [6]. The α -adaptin subunit of AP2 is expressed in humans as a full-length transcript in forebrain, skeletal muscle, spinal cord, cerebellum, salivary gland, heart and colon [32]. There are two alpha-adaptin genes, alpha A and alpha C [5]. During translation the alpha A mRNA is alternatively spliced resulting in two protein isoforms that are found in most tissues but which are different from the alpha C expressed in brain. The shorter alpha A isoform is missing 21 amino acids within the hinge region, giving it a similar electrophoretic mobility to that of alpha C [5]. The mRNA of the shorter alpha A isoform and alpha C are found in heart tissue [5].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor super family and play pivotal roles in the regulation of energy metabolism [15]. PPARs consist of three different isotypes α , β , and γ , which in turn heterodimerize with α -, β -, and γ -isotypes of the 9-cisretinoid X receptor (RXR) for binding to peroxisomal proliferator response elements (PPREs) in the promoter regions of target genes involved with energy metabolism [10]. Functional PPREs are canonically defined as a direct repeat-1 (DR1, DR2) response element composed of two divergent half sites (AGGTCA) separated by either one or two nucleotide [18]. The RAR/RXR-REs are defined as a direct repeat response element composed of two half sites (TGACCT) [20] which are the complement DNA sequence of the canonical PPREs. A recent study coalesced the great diversity of PPREs in the promoter regions of various genes [19] indicating the degeneracy of these elements through evolutionary time and speciation [3, 14]. This degeneracy is most likely results from the inability of PPARs to bind response elements by themselves and the required heterodimerization with the retinoid receptors whose response elements requirements have been more conserved through evolution [4, 7, 10].

Although much is known about PPARs function and involvement in gene expression, little is known about its regulation of genes in various organs. In this study we report on the up regulation of the mouse AP2 α 2 gene in heart tissue by PPAR α through the use of microarray technology, quantitative real time PCR, computer analysis of the gene's promoter region, electrophoretic mobility shift assay (EMSA) and transactivation assays. Our details and results of the microarray analysis have previously been reported for putative target genes of PPAR α [8]; however, a further examination of the regulation of AP2 α 2 by PPAR α merits confirmation.

2 Materials and Methods

2.1 Animals, PPAR α Activation and Microarray Methods

The 8337T TR β transgenic mouse use in this study was created to reproduce the human genetic disease known as resistance to thyroid hormone (RTH) [1, 26]. The use of this transgenic mouse, Wy14643 drug activation of PPAR α target genes and microarray methods have been previously reported [8]. In brief, mice expressing the cardiac specific 8337T mutation and non-transgenic littermates were sacrificed 6 h after intra-peritoneal injection of Wy14643 (50 mg/kg). The hearts were extracted and prepared for mRNA expression studies. The evidence for PPAR activation of the AP2 α 2 gene using microarray analysis is presented in Table 1. All animal studies were performed in accordance with guidelines from Seattle Children's Research Institute Animal Care Committee approval and conforming to National Institutes of Health guidelines.

2.2 Q-RT PCR (Real-Time Quantitative PCR)

Q-RT PCR was conducted with products obtained from SuperArray Bioscience Corporation, Frederick, MD, including the first strand cDNA synthesis application. PCR was carried out using HotStart DNA Taq polymerase and SYBR green/fluorescein master mix containing all reagents and buffers required for q-PCR on the iCycler PCR machine (Bio-Rad). The mAP2 α A (NM_007459) primer sets (PPM04666A) obtained from SuperArray generated a 162 bp amplicon that was confirmed by agarose gel electrophoresis and ethidium bromide staining. All procedures were run according to the instructions provide by the manufacturer.

2.3 Computer Analysis of Promoter Sequences

The Vector NTI Advance 11 computer program from Invitrogen, Carlsbad, CA, was use to survey the promoter region of the mAP2 α A gene from -5,000 bp upstream of the

transcriptional start site (TSS) to 2,500 bp downstream for the PPRE AGGTCA/TCCAGT motif sequences. The results of the survey are presented in Table 2. The promoter regions of four other genes of interest which have documented PPREs were also surveyed for the AGGTCA/TCCAGT motif sequences and included (Table 2).

2.4 Transactivation Assay

HepG2 cells were co-transfected by TfxTM-20 (Promega) with mPPAR α (m is mouse), mPPAR β or mPPAR γ ₁ expression vector and pGL4 reporter vector containing different size fragments of the hAP2 α 2 (h is human) promoter. All hAP2 α 2 plasmid constructs were sequenced and compared with the gene promoter region (NM_012305) for PCR accuracy. The *Renilla* reporter vector was co-transfected to normalize for differences in transfection efficiency. After transfection, cells were incubated in the presence or absence of Wy14643 (50 mM) for 4 h before lysis. A Promega dual-luciferase reporter assay was used to measure the relative promoter activities. The transactivation differences within each pGL4 construct for mPPAR α /RXR α transfections with and without Wy14643 were tested using a student's unpaired *t* test.

2.5 Electrophoretic Mobility Shift Assay (EMSA)

The hRXR α , hPPAR α and hPPAR γ proteins were generated from pSG5 expression vectors using the transcription and translation (TNT) coupled in vitro system (Promega, Madison, WI). The following oligonucleotides were annealed: AP-2 α A-PPRE, 5'-AGCAAGTTTGAGGTCAGCCTGAGCCA-3' and 5'-GTTCAAACCTCCAGTCGGACTCGGTGTAC-3'; AP-2 α A-PPREmut, 5'-AGCAAGTTTGAGGACAGCCTGTGCCAC-3' and 5'-GTTCAAACCTCCTGTCTGGACACGGTGTAC-3'; for specific competition malic enzyme PPRE, 5'-GGACTTTCTGGGTCAAAGTTGATCCCCC-3' and 5'-CCTGAAAGACCCAGTTTCAAACCTAGGGGGAG-3'; and for non-specific competition Ets, 5'-TGGAATGTACCGGAAATAACACCA-3' and 5'-ACCTTACATGGCCTTTATTGTGGT-3'. Oligonucleotides were annealed and labeled by Klenow filling enzyme (Promega) using Redivue [α -³²P]dCTP (3,000 Ci/mmol) (Amersham Biosciences, Piscataway, NJ). In vitro translated proteins (1 mL per reaction) were pre-incubated for 15 min on ice in 1 $\times$ binding buffer [80 mM KCl, 1 mM dithiothreitol, 10 mM Tris/HCl (pH 7.4), 10% (v/v) glycerol plus protease inhibitors] in the presence of 2 μ g of poly (dI-dC) (dI-dC), 5 μ g of sonicated salmon sperm DNA and competitor oligonucleotides in a final volume of 20 μ L. Then 1 ng (1 ng/ μ L) of radiolabelled oligonucleotide was added, and incubation proceeded for another 10 min at room temperature (25 °C). Complexes were separated on a 4% polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) equilibrated in 0.5 $\times$ TBE (Tris/borate/EDTA) at 20 mA.

2.6 Immunoblotting

Fifty micrograms of total protein extract from mouse heart tissue were electrophoresed along with two lanes of molecular weight size markers (chemichrome western control, Sigma) in 4.5% stacking and a 10% resolving SDS-polyacrylamide gel. The gels were then electroblotted onto PDVF plus membranes. The western blot was blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline plus Tween-20 (TBST) [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20], followed by overnight incubation at 4 °C with a AP2 α 2 mouse monoclonal primary antibody (sc-55497, Santa Cruz Biotechnology, Inc.) diluted in the above blocking solution. After two 10-min washes with TBST and one 10-min wash with Tris-buffered saline (TBS), the membrane was incubated at room temperature for 1 h with a bovine anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (HRP) (sc-2371, Santa Cruz Biotechnology, Inc.). The membranes were washed twice for 10 min with TBST and visualized with enhanced chemiluminescence

after exposure to Kodak biomax light ML-1 film. The membrane was stripped by washing two times for 30 min with 200 mM Glycine, 0.1% SDS and 1% Tween-20 (pH adjusted to 2.2), followed by three 10-min washes with TBS. The membrane was again blocked for 1 h as above, followed by overnight incubation at 4 °C with a GAPDH rabbit polyclonal antibody (sc-25778) diluted 1:200 in blocking solution. The next day the membrane was washed (as above), a goat anti-rabbit secondary antibody-HRP (sc-2313) was applied, and the remaining procedure as described above was followed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference to verify protein lane loadings.

2.7 Immunofluorescence for AP2α2 Detection in Cardiomyocytes

Freshly prepared cardiomyocytes extracted from neonatal rat heart tissue were plated onto two 400 μL wells on a glass coverslip (0.17 mm thick no. 1.5) at a cell density of 2.5×10^4 cells/400 μL neo cardio media [13] and placed in 37 °C, 5% CO₂ incubator over night. The next day the media was aspirated and neonatal cardiomyocytes were treated with either 1% DMSO and neo cardio media or 50 μM Wy14643 in 1% DMSO and neo cardio media then placed back in the incubator for 24 h. After a 24 h incubation period, cardiomyocyte movement or cell viability was visually detected under a microscope. A distinct beating pattern, characteristic of healthy cardiomyocytes, was observed by two of authors (NEB and HR). The drug treated media was aspirated from the viable cells and Minimal Essential Media (MEM) was added to the cells. The cells were allowed to incubate for another 30 min and then used to detect changes in AP2α2 activity between DMSO and Wy14643 treated cells. Since we hypothesized that PPARα regulates the AP2α2 gene, any changes in AP2α2 activity in the Wy14643 treated cells would be the result from PPARα activation by its agonist.

To differentiate cardiomyocytes from fibroblasts, the cells were placed on ice for 15 min to stop endocytosis then moved to RT and fixed with a fresh 4% paraformaldehyde solution for another 15 min. The fixed cells were treated with a solution (1:1,000) of fluorescein-conjugated phalloidin (SKU# F432, Molecular Probes) in MEM media for 15 min at RT and washed three times for 5 min. The slides were examined under a fluorescence microscope with appropriate filters to differentiate cardiomyocytes from fibroblasts (data not included).

For AP2α2 detection the cells were placed on ice for 15 min to stop endocytosis and then moved to RT and fixed with a fresh 4% paraformaldehyde solution for another 15 min. The cells were rinsed three times for 5 min with PBST (phosphate-buffered saline that contained 0.05% Tween-20) and then blocked (1:10) with normal goat serum in PBST for 5 min. The cells were briefly rinsed with PBST and then incubated in a solution (1:50) containing the AP2α2 mouse monoclonal primary antibody (sc-55497, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and PBST over night at 4 °C. The next day the cells were rinsed three times for 5 min with PBST and then incubated in a solution (1:100) of the goat anti-mouse IgG, F(ab)₂-PE-Cy5 secondary antibody (sc-3799, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and PBST for 30 min at RT to analyze for immunofluorescence cardiomyocyte AP2α2 staining. The cells were then rinsed two times for 5 min with PBST and once with DDI water. The cells were then mounted with two drops of Prolong Gold Anti-fade media containing DAPI (Molecular Probes/Invitrogen) and allowed to cure in the dark at RT overnight. The slides were stored at -20 °C until examined by a fluorescence microscope with appropriate filters. Z-axis stacks were taken with a Leica DC500 camera mounted on a Leica DMI6000B microscope using a 63× oil objective. Out of plane light was removed using Autoquant X2 deconvolution software (MediaCybernetics), and single images in the same Z-plane as the nucleus were chosen for publication. Phase-fluorescence image overlays were created using ImagePro v6.3 (MediaCybernetics, Bethesda, MD). Co-localization analysis was performed on corresponding optical slices by the JACoP plugin

(Publicly available freeware, <http://rsb.info.nih.gov/ij/plugins/track/jacop.html>) in ImageJ v1.43 (NIH freeware, <http://rsbweb.nih.gov/ij/>).

3 Results

3.1 Wy14643 Induces PPAR α to Activate and Up-Regulate the AP2 α 2 Gene

Microarray analysis of control and 8337T TR transgenic mice treated with the PPAR agonist Wy14643 identified the AP2 α 2 gene as a potential PPAR α target gene [8]. Unpublished microarray data from that study indicated that the gene expression was up regulated by PPAR α in both the control and transgenic mouse (Table 1). In order to confirm the microarray results, we conducted q-RT PCR analysis on the mRNA levels in the Wy14643 treated and untreated mouse groups (Fig. 1). The q-RT PCR results indicated that the treated control and transgenic mice were significantly up regulated over the untreated mouse groups by PPAR α .

3.2 PPRE Identified in the Promoter Region of the AP2 α 2 Gene

To verify that PPAR α regulates the AP2 α 2 gene, we conducted a computer analysis search for the PPRE AGGTCA/TCCAGT motif sequence in the promoter region of this gene (Table 2). The search was made from –5,000 bp upstream of the transcription start site of the gene to 2,500 bp downstream from the site using the Vector NTI Advance 11 computer program. We identified five AGGTCA/TCCAGT motif sequences in the mouse and four in the human promoter regions that could serve as possible PPRE sites. In order to identify which of these sequences is the punitive PPRE site, we conducted gel shift and transactivation assays (Figs. 2, 3). In the presence of PPAR α or RXR α only, a single complex was observed, which originated from the reticulocyte lysate (Fig. 2). An additional, more intense, slower moving complex was observed only in the presence of both receptors, indicating that it represents a PPAR-RXR heterodimer. This complex disappeared in the presence of an excess of unlabelled specific oligonucleotide, but not for the non-specific oligonucleotide (i.e., the complex was weak but still present in a 100-fold excess of non-specific oligonucleotide). The PPAR-RXR heterodimer did not form on a mutated oligonucleotide that contained two substitutions within the AP2 α 2 PPRE. Similar results were observed for the PPAR γ (Fig. 2). These results indicate that PPAR α and γ are able to bind to the –2,930 bp AGGTCA/TCCAGT motif sequence in vitro and thereby establish the motif as a punitive mAP2 α 2 PPRE. This motif was verified as a PPRE when luciferase transactivation assays were conducted on hAP2 α 2 (Fig. 3). To determine which promoter region is responsible for the PPAR-induced up-regulation of the AP2 α 2 expression 1, 2.2 and 3.2 kb fragments of the hAP2 α 2 promoter immediately upstream of the TSS were cloned in front of a luciferase reporter and transactivation studies were carried out in HepG2 cells (Fig. 3a). The PPAR α agonist Wy14643 increased reporter activity significantly ($p < 0.05$) in the 3.2 kb pGL4 construct while showing no significant differences in the other constructs in spite of an apparent increase in reporter activity for the –912 bp construct (Fig. 3a). The increase in report activity for the 912 bp construct could not be verified when a gel shift assay was run on the AGGTCA/TCCAGT motif sequence at –560 bp from the TSS. This suggests that the active PPRE is located between 2.2 and 3.2 kb upstream of the TSS which supports the gel shift data (Fig. 2). In this region a TGAGGTCAG sequence was identified that is conserved between the human and mouse AP2 α 2 promoter, suggesting that it is important for regulation (Fig. 3b). Although the PPAR α activator Wy14643 was the only agonist used in this study, we found that it evidently activates PPAR β because it also increased reporter activity in the 3.2 kb pGL4 construct with very similar results found for PPAR α , while PPAR γ displayed no change in reporter activity (Fig. 3c). This is perhaps due to the fact that the Wy14643 agonist has not been reported as a ligand for mPPAR γ .

3.3 AP2 α 2 Protein Levels

In addition to analyzing mRNA levels in non-treated and Wy14643 treated mice, the relative steady-state AP2 α 2 protein level was also examined by western blot analysis (Fig. 4). This analysis indicates that the AP2 α 2 protein levels remain the same between the control and Wy14643 treated mice after the single 6 h drug treatment suggesting a slow or non-existing translational response or that the protein response was outside the window of sampling.

3.4 An Increase in AP2 α 2 Detected in Wy14643 Treated Cardiomyocytes

Immunofluorescent detection of AP2 α 2 in cultured rat cardiomyocytes revealed two differing localization patterns: (Fig. 5a) distribution in the nucleus and (Fig. 5b) nuclear localization, with an additional distribution dispersed throughout the cytoplasm. An increase in AP2 α 2 activity is apparent in cardiomyocytes treated for 24 h with Wy14643 (Fig. 5b) as compared to the DMSO treated cells (Fig. 5a). This observed localization pattern is further supported by a difference in co-localization between DNA and AP2 α 2 in the two treatment groups. Mander's and Pearson's colocalization coefficients for the DMSO treated cells were 0.733 and 0.742, respectively. These numbers dropped to 0.501 and 0.507 for the Wy14643 treated cells, suggesting that AP2 α 2 localization shifts from the nucleus to the cytoplasm upon drug treatment. In this experiment, activity of the AP-2 complex is defined by its intracellular localization.

4 Discussion

Using microarray [8] and qRT-PCR technology, we identified the AP2 α 2 gene as being differentially expressed between hearts of control or δ 337T TR β transgenic and Wy14643 treated mice. Although it was not necessary to include the transgenic mouse in this study, we elected to include the data as a follow up to our previous work [8]. The follow-up analysis subsequently revealed that AP2 α 2 is a direct target gene of PPAR α and probably PPAR β and PPAR γ . A functional PPRE has been identified in the human and mouse AP2 α 2 promoter 2.9 kb upstream from the transcriptional start site.

We analyzed the promoter region for the AGGTCA/TCCAGT motif sequences between -5,000 bp upstream to 2,500 bp downstream from the TSS for the AP2 α 2 gene as well as four other genes with known PPRES (Table 2). In addition to the -2,930 bp PPRES for AP2 α 2, four other AGGTCA/TCCAGT motif sequences were identified in the human and five in the mouse promoter regions. For the mouse G α /G β 1 switch gene 2 (mGOS2) there were 11 additional AGGTCA/TCCAGT motif sequences found by computer analysis in addition to the one reported in mGOS2 PPRES (38). For the human uncoupling protein-3 (hUCP3) gene, there were seven additional AGGTCA/TCCAGT motif sequences found as well as one in each of the three reported PPRES for this gene [2]. For the human acyl coenzyme A oxidase gene (hACOX1), there were seven additional motif sequences found in addition to the one found in the reported PPRES [35]. For the mouse phosphoenolpyruvate carboxykinase gene (mPCK1), there were five additional motif sequences found as well as the one in the reported PPRES [33].

This pattern suggests there maybe multiple PPRES that operate in the promoter regions of genes regulated by the PPARs as shown to be the case for hUCP3 [2]. This was recently eluded to when the PPRES for the promoter regions of the mGOS2 [38] and m/h I κ B α [7] genes were identified. In this study we identified one PPRES motif (AGGTCA/TCCAGT) while there are other PPRES motifs that may be involved in AP2 α 2 gene regulation by the PPARs [19].

AP-2 is characterized as a member of heterotetrameric clathrin adaptor complexes that play pivotal roles in many vesicle trafficking pathways within the cell [9]. Clathrin adaptor AP-2

is involved with trafficking of the MHC-II molecules to antigen-processing compartments [21], of the protease-activated receptor 1 (PAR1), a G protein-coupled receptor for the coagulant protease thrombin [25] and early embryonic development [22]. It is not surprising that the AP2 α 2 gene with its involvement in trafficking has been identified as a novel PPAR target gene since this group of nuclear and mitochondrial receptors are strongly associated with energy metabolism and lipid transport.

The intracellular localization of the rat cardiomyocyte AP2 α 2 subunit after 24 h incubation with DMSO was observed to be nuclear, as ascertained by deconvolution fluorescent microscopy. In contrast, incubation of rat cells with Wy14643 for 24 h resulted in not only a pronounced nuclear localization but a widespread cytoplasmic distribution that included punctuated signals at the plasma membrane. Our interpretation of these observations is that the gene expression of the AP2 α 2 subunit is up-regulated throughout the 24 h incubation with Wy14643 to levels that result in continued synthesis and sorting to the plasma membrane. Continual endocytosis of undefined cargo would then be linked to continual trafficking across the nuclear membrane into the nucleoplasm with subsequent degradation or recycling back to the cell surface. Our analysis of the AP2 α 2 primary structure failed to identify a nuclear localization signal, raising the possibility of nucleocytoplasmic transport receptors such as the importins may be involved in translocating the AP2 α 2 subunit across the nuclear membrane and into the nuclear space. Although the no nuclear localization signal could be identified in our study, other reports indicate the occurrence of clathrin adaptor proteins in the nucleus. The clathrin adaptor α -adaptin, but not β -, μ 2, or γ -adaptins, accumulates in the nucleus of HeLa and CV-1 cells upon treatment with leptomycin B [36] which suggests a nuclear transport system. Comparative genomic evidence for the nuclear pore complex indicates that clathrin proteins have evolved as part of that complex [23]. Also, it has been shown that clathrin is bound to microtubules or microtubule-associated proteins in the stabilization of kinetochore fibres of the mitosis spindle apparatus [30]. Our in vitro data is supported by our in vivo murine qt-PCR data which also indicates an up-regulation of AP2 α 2 gene expression. The western blot data is a measure of total mouse heart tissue AP2 α 2 protein and therefore does not reflect any changes in intracellular localization. The collective evidence points to a continual cycling of the AP2 α 2 subunit from the ribosome to the cell surface with subsequent sorting to the nuclear membrane. Since the shorter alpha A and alpha C isoforms are both found in heart tissue [5], it is possible that one isoform localizes to the cytoplasm and one to the nucleus. In conclusion, we have identified AP2 α 2 as a novel direct target gene of PPAR α , and probably PPAR β and PPAR γ due to the evidence presented here involving transactivation and gel shift assays, respectively.

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Abbreviations

PPAR	Peroxisome proliferator-activated receptor
RE	Response element
PPREs	Peroxisomal proliferator-activated receptor response elements
AP2α2	Adaptor-related protein complex 2, alpha 2 subunit
RXR s	Retinoid X receptors

DMSO	Dimethyl sulfoxide
Wy14643	PPAR activator
TRβ	Thyroid hormone receptor beta
6337T TRβ	Deleted thymine at amino acid 337 in thyroid hormone receptor
HepG2	Liver tissue culture cell line
CCVs	Clathrin-coated vesicles
CME	Clathrin-mediated endocytosis
TSS	Transcriptional start site

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Fig. 1.

Cardiac expression of AP2 α 2 after Wy14643 treated and untreated control and δ 337T TR β transgenic mice. There were seven mice per group. Treated mice received an injection with a mix of saline, DMSO and Wy14643 and control mice received an injection of vehicle (DMSO) and saline. Hearts were extracted 6 h after treatment and AP2 α 2 expression was determined by Q-RT PCR. Results are means $\pm$ SEM. Differences were evaluated by student's t test (* p <0.05, **<0.01)

Fig. 2. Binding of the PPAR-RXR heterodimer to the putative AP2α2 PPRE as determined by gel shift assay. A double-stranded labeled oligonucleotide containing the AP2α2 PPRE was incubated with in vitro transcribed/translated hRXRα and hPPARα (*left side of the panel*) or hPPARγ (*right side of the panel*), and binding complexes were separated by electrophoresis. Fold-excess of specific (Spec: malic enzyme PPRE) or non-specific (Non-sp: Ets oligonucleotide) unlabelled probe is indicated

Fig. 3.

a hAP2α2 reporter constructs containing 912, 2,156 and 3,187 base pairs of immediate upstream promoter region were transfected into HepG2 cells together with mPPARα expression vector. Transfected cells were incubated for 4 h in the presence or absence of the agonist Wy14643. A luciferase reporter containing three copies of the acyl-CoA oxidase PPPE was used as a positive control. Normalized luciferase activity of the 912, 2,156 and 3,187 bp reporters in the absence of PPARα and Wy14643 were set at one. Results are means ± SD for three independent experiments. **b** Alignment of a putative TGAGGTCAG regulatory region within hAP2α2 and mAP2α2 promoter about 2.9 kb upstream from the transcription start site. **c** hAP2α2 reporter construct containing 3,187 bp of immediate upstream promoter region was transfected into HepG2 cells together with a PPAR expression vector. Transfected cells were incubated for 48 h and then for an additional 4 h in the presence or absence of Wy14643. Normalized luciferase activity in the absence of PPAR and ligand was set at one. Results are the means ± SD for three independent experiments

Fig. 4.

Representative Western blots and relative protein expression for wild-type (C) and wild-type +Wy14643 (CW) with a 6 h drug exposure (see Sect. 2.1, $n = 7/\text{group}$). Protein expression is expressed relative to GAPDH to account for protein loading differences per lane.

Accompanying blots show mAP2 α 2 protein at *top* and GAPDH at *bottom*

Fig. 5. Immunofluorescent detection of: (A & B) AP2α2, comparing Wy14643 treated and untreated cells. Images were collected using a DMI6000B inverted fluorescence microscope (Leica Microsystems, Bannockburn, IL), a 63× oil immersion planapochromatic objective (NA = 1.32), and a Leica DC500 digital camera (1,300 × 1,030 pixel resolution). Cells were optically sectioned by collecting a z-stack of images at focal planes 0.3 μm apart. The z-stack collection was automatically controlled by ImagePro v6.3 software and deconvoluted with Autoquant X. Size bar = 15 μm

Table 1

Two unpublished AP2α2 Affymetrix mouse mRNA array expression results obtained from an Affymetrix mouse expression 430_2 gene chip array study [8]

Gene symbol	Representative public ID	Log 2_mean control	Log 2_mean contl + Wy14643	Log 2_mean δTR337	Log 2_mean δTR337 + Wy14643	Permutation <i>p</i> value Wy14643 effect	Drug fold change
AP2α2	AK009735	5.833	6.292	5.797	6.448	0.001	1.469
AP2α2	AK009735	4.626	4.809	4.684	4.891	0.008	1.145

Listed are the AP2α2 log2 mean expression intensities of seven mouse hearts per group, permutation *p* values of drug effect (mouse vs. drug) which shows a significant difference between untreated and treated mice with the PPARα agonist Wy14643 ($p < 0.05$). Also listed are the fold changes (Wy14643 treatment/untreated) where a value greater than one indicates up-regulation of AP2α2

Table 2

PPAR-RE and RAR-RE sites within the mouse (m) or human (h) promoter regions of five genes. The response elements (REs) in the promoter regions for the mGOs2, hUCP3, hACOX1 and mPCK1 genes have previously been documented while the RE for the AP2α2 gene is from this study

Gene symbol	RefSeq ID	<u>PPAR/RAR(AGGTCA/TCCAGT)</u>		Response elements Sequence (5' to 3')	RE-Ref
		# of sites	Location from start site		
mAP2α2	NM_007459	5	-2,930	AGCAAGTTTG AGGT CAGCCTGAGCCA GTTCAA ACTTCCAGT CGGACTCGGTGTAC	This study
hAP2α2	NM_012305	4	-2,930	GGGCGGATCATG AGGT CAGGAGATTGAGAC CCCGCCTAGTACT TCCAGT CCTCTAACTCTG	This study
mGOs2	NM_008059	12	-1,796	TGCATCAGT AGCTTT GCAATTTTCTGG GTC ACTTGGAA ACGTTAAAGACCGGTC	28
hUCP3	NM_022803	10	-3,519	TGACATTA AGGTCA	29
				ACTGTAAT TCCAGT	
			-2,305	TTGACCTTT GGA CT	29
				AACTGGAA ACCTGA	
hACOX1 Isoform A	NM_004035	8	-2,084	AGGTTTC AGGTCA	29
				TCCAAAG TCCAGT	
			-1,927	CTTAGAACTAGAA AGGTCA CTGGTCAAGCAGCCATTT GAATCTTGATCT TCCAGT GACCAGTTCGGTAAACC	30
mPCK1	NM_011044	6	-766	CCTTCTCA TGACCTTT GGCCGTGGGAG	31
				GGAAGAGT ACTTGGAA ACCGGCACCCTC	

Listed are the number of PPAR/RAR sites found between -5,000 and 2,500 bp of the transcriptional start site of each gene. The PPAR/RAR sites were obtained using the Invitrogen Vector NTI Advance 11 program