Identification of the CREB-binding Protein/p300-interacting Protein CITED2 as a Peroxisome Proliferator-activated Receptor α Coregulator*§

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Like other nuclear receptors, the peroxisome proliferator-activated receptors (PPARs) use a wide variety of protein-protein interactions to properly regulate transcription of target genes. In an attempt to identify novel PPAR-interacting proteins, a cDNA expression library was screened with bacterially expressed PPARα. One of the genes identified as a PPARα-associated protein by interaction cloning was the CREB-binding protein/p300-interacting transactivator with ED-rich tail 2 (CITED2, also called p35sr/mdrg1/msgl1). This coactivator interacted directly with PPARα in the presence or absence of ligand predominantly via the ligand binding domain of the nuclear receptor. In transient transfection reporter assays, CITED2 acted as a dose-dependent coactivator of PPARα-dependent transcriptional regulation in the presence of several exogenous ligands. CITED2 also increased PPARγ-dependent regulation of reporter genes but had no effect on PPARβ activity. To determine whether CITED2 affects endogenous gene expression, this protein was stably overexpressed (CITED2+) or repressed by small inhibitor RNA (CITED2−) in immortalized mouse hepatocytes. Relative to the control stably transfected or CITED2− cells, CITED2+ cells had an increased rate of cell proliferation. Microarray analysis and real time PCR showed that several genes are differentially affected by PPARα ligands in CITED2+ versus CITED2− cells. Genes that were affected by PPARα ligands in a CITED2-modulatory manner include angioptatin-like protein 4, forkhead C2, hypoxia-inducible factor-1α, and MAPK phosphatase 1. Interestingly these genes share common functions in that they are known to promote vascularization and angiogenesis in response to hypoxia. The results described here suggest that CITED2 is a coactivator of PPARα and that both proteins may participate in signaling cascades of hypoxic response and angiogenesis.

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The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor (NR) superfamily and exist as three distinct subtypes (α, β/δ, and γ; NR1C1–3 (1)). The receptors, so named for their ability to respond to a class of compounds deemed peroxisome proliferators, have been implicated in a wide range of biological processes such as cell growth, lipid homeostasis, apoptosis, and response to xenobiotics and endogenous compounds. In particular, PPARα has a role in carcinogenesis with peroxisome proliferators being potent hepatocarcinogens in rodents (for a review, see Ref. 2).

The regulation of gene expression by PPARs follows the classic NR mechanism whereby the receptor binds to ligand, translocates to the nucleus, heterodimerizes (retinoid X receptor α), binds to a conserved response element (DR1) in the promoter region of the target gene, and regulates transcription. One aspect of this mechanism that has drawn increasing attention is the potential protein-protein interactions that lead to the proper regulation of the target gene. Several proteins, often called coregulators, have been identified in the nuclear receptor transcription complexes that affect target gene expression. The family of coregulator proteins can be divided into two basic classes, coactivators that serve to increase the level of transcriptional activation of a gene and corepressors that do the opposite. These proteins come in many forms and may or may not contain enzymatic activity such as histone acetyltransferase (e.g. CREB-binding protein (CBP)/p300) or histone deacetylase (e.g. nuclear receptor co-repressor). Those coregulators that do not contain enzymatic activity are thought to serve as a protein bridge that span the distance between the transcription factor and the elements of the complex that act directly upon the target gene. It is these bridging proteins that serve to further regulate transcription by helping to create distinct transcriptional complexes in response to particular chemicals whether endogenous or exogenous. Many coactivators have been initially isolated as PPAR coactivators such as PPAR-binding protein (3), PPARγ coactivator-1 (4), and PPAR receptor interaction protein (same as thyroid receptor-binding protein (5)).

The CBP/p300-interacting transactivator with ED-rich tail (CITED) family of proteins contains four members to date. This class of protein has a highly acidic carboxyl-terminal end that..
interacts with CBP/p300. The CITED proteins affect the transcriptional activity of many transcription factors ranging from AP2 (6), estrogen receptor (7), and hypoxia-inducible factor 1α (HIF1α) (8). One member of this family, CITED2 (also called p35srj, mrg1, and msg1), is involved in the transcriptional repression of HIF1α (8). Previous studies have suggested that CITED2 plays a role in the development of the nervous system since the CITED2−/− mouse died in late gestation and showed malformation in the mid and hind brain (9). CITED2 can repress INK4a/ARF via regulation of Bmi1/Mel18 leading to decreased cell proliferation (10). This is significant since the main phenotype of the CITED2−/− mouse is defects in neural tube closure that is thought to be caused by a loss of cell proliferation and increased cell death.

In these studies, CITED2 was identified as a PPARα-associated protein in an interaction cloning screen. Due to the role of CITED2 as a coactivator and a CBP/p300 bridging factor, we hypothesized that it would affect the transcriptional activity of PPARα. Using a variety of in vivo and in vitro assays, we demonstrated that CITED2 directly interacts with PPARα and acts as an exogenous ligand-dependent and -independent coactivator. Also we demonstrated that CITED2 can affect cell cycle regulation and gene expression in hepatocytes in the absence of exogenous PPARα activators.

EXPERIMENTAL PROCEDURES

Materials—Wy-14,643 (4-chloro-6-(2,3-xylindino)-2-pyrimidinylthio)-acetic acid, Chemical Abstracts Society number 50892-23-4 (<98% pure) was purchased from Chemsyn Science Laboratories (Lenexa, KS). DH5α bacterial cells were purchased from PGC Scientific (Frederick, MD). Media components were from Invitrogen. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT). Bovine serum albumin, linoleic acid (CLA) for 6 h. Luciferase activity was determined and corrected for transfection efficiency and extraction yield. Each domain and treatment group was corrected to their corresponding VP16 value (100%). * p < 0.01 comparing VP16 bar to corresponding VP16 bar. The graph is representative of three independent experiments.

CITED2 Is a Coactivator of PPARα

VP16—CITED2

![Graph showing the interaction between CITED2 and PPARα](https://example.com/graph.png)

**Fig. 1.** Verification of the interaction between rat PPARα and CITED2 in vitro. CITED2 was radiolabeled with [35S]methionine and incubated with bacterially expressed PPARα-MBP fusion for 1 h at 4 °C in the presence of amyllose resin. Resin was collected and washed three times with cold radioimmune precipitation assay buffer. Bound MBP was eluted from the resin using 10 mM maltose in radioimmune precipitation assay buffer for 1 min at 4 °C. Eluate was resolved on a 12% Tris-glycine gel, dried, and subjected to autoradiography. The image is representative of two independent experiments.

COS-1 cells were transiently transfected with the GAL4-DBD fused to rPPARα and VP16 activation domain with or without fused CITED2. Cells were treated with 50 μM Wy-14,643 (Wy), 200 μM CLA mixture, or MeSO (DMSO) for 6 h. Domains containing no ligand activation were treated with MeSO. Luciferase activity was determined and corrected for transfection efficiency and extraction yield. Each domain and treatment group was corrected to their corresponding VP16 value (100%). * p < 0.01 comparing VP16 bar to corresponding VP16 bar. The graph is representative of three independent experiments.
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Fig. 3. CITED2 is a coregulator for PPARα. A, HepG2 cells were transiently transfected with expression vectors for rPPARα with CITED2 or empty vector control (pcDNA3) and a PPRE-driven luciferase reporter. B, COS-1 cells were transiently transfected with rPPARα fused to the GAL4-DBD and CITED2 or empty vector control with GAL4-responsive reporter. Cells were treated with 50 μM Wy-14,643 (Wy), 200 μM CLA mixture, 100 μM ciprofibrate, or MeSO (DMSO) for 6 h. Luciferase activity was determined and corrected for transfection efficiency and extraction yield. All bars are corrected to untreated pcDNA3 level. *, p < 0.05 comparing CITED2 bar to corresponding pcDNA3 bar. The graph is representative of three independent experiments.

**Mouse Oligonucleotide Arrays**—The Mouse Genome Oligo Set Version 1 was purchased from Operon (Alameda, CA) and contains 6800 optimized 70-mers plus 24 controls with melting temperature normalized to 78°C. Sequences were optimized by the manufacturer using BLAST against all known mouse genes to minimize cross-hybridization. Oligonucleotides were printed onto glass slides using GeneMachines Omnigrid (San Carlos, CA) with additional controls obtained from Stratagene (SpotReport system, Stratagene, La Jolla, CA) at the Pennsylvania State University microarray core facility.

**Microarray Analysis**—Total RNA was isolated by TriReagent (Sigma) and further purified with RNAEasy (Qiagen) according to the manufacturers’ instructions. Details of the microarray analysis including reverse transcription, labeling, and hybridization can be found elsewhere (13). In the present experiments, cohybridization was performed with cDNA from CITED2 + treated cells labeled with Cy5 and those from control-transfected samples labeled with Cy3. Scanning was performed by a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA), and image acquisition was performed with Axon GenePix image software. Analysis of gene expression data was performed using GeneSpring software (Silicon Genetics Inc., Redwood City, CA) and PathwayAssist (Ariadne Genomics, Rockville, MD) (see supplemental data).

**Statistical Analysis of Microarray**—Normalization and analysis of the gene expression profiles were performed as described previously (13). Intensity-dependent normalization was also applied where the ratio was reduced to the residual value of the Lowess fit of the intensity ratio curve. Statistical analysis was performed using a Student’s t test with a p value of 0.05 with the additional criteria of being either 1.5-fold increased or decreased in CITED2 + versus control-transfected cells. Genes that met these parameters were classified by molecular function using annotations from Mouse Genes or Ariadne Genomics.

**Real Time PCR**—Total RNA was isolated as before. The total RNA was reverse transcribed using the ABI High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Standard curves were made using serial dilutions from pooled cDNA samples. Real time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol and amplified on the ABI Prism 7000 sequence detection system.

**RNA Inhibitor Design**—A double-stranded RNA inhibitor for CITED2 was designed using the sequence from GenBank. The sequence was synthesized and cloned into the pSUPER.neo plasmid (Oligoengine, Seattle, WA). All transfections of RNAi plasmids were performed using LipofectAMINE (Invitrogen) transfection reagent according to the manufacturer’s protocol. The sequence chosen for the CITED2 RNAi is as follows: 5′-CCACTACATGCCGGATTGTG-3′.

**Western Blot Analysis**—Total cell protein was isolated using cell lysis solution (50 mM HEPES, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM EDTA, 1 mM NAF, 10 mM β-glycerophosphate, 0.1% protease inhibitor mixture (Sigma), 0.1 mM sodium orthovanadate) and lysing on ice for 30 min followed by centrifugation at 12,000 × g for 10 min. The supernatant was removed as total soluble protein. Total soluble protein (50 μg) was separated on a 12% Tris-glycine gel and electrotransferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore). Membranes were washed three times with TBS, 0.1% Tween 20 (TBS+), blocked with 5% nonfat dry milk in TBS+ for 1 h at room temperature, washed three times with TBS+, and incubated while rocking at room temperature for 1 h with primary antibodies. Immunoblotting was performed using a mouse anti-CITED2 antibody (Novus Biologicals, Littleton, CO) in TBS+, 0.5% dry milk. The blot was washed three times with TBS+ and incubated at room temperature for 1 h with anti-mouse (Amersham Biosciences) at 1:10,000 dilution in TBS+. 0.5% dry milk. The blot was then washed three times with TBS+, and visualization was performed using enhanced chemiluminescent visualization (ECL, Amersham Biosciences).

**Cell Growth Analysis**—Cells of interest were plated at 200 cells/well in a 96-well plate, allowed to recover overnight in normal defined agarose gel (Cambrex) and quantified by densitometry.

**Normalization and Analysis of Microarray Data**—Normalization and analysis of gene expression data was performed using the GeneSpring software (Silicon Genetics Inc., Redwood City, CA) and image acquisition was performed with Axon GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA), and image acquisition was performed with Axon GenePix image software. Analysis of gene expression data was performed using GeneSpring software (Silicon Genetics Inc., Redwood City, CA) and PathwayAssist (Ariadne Genomics, Rockville, MD) (see supplemental data).

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Identification of CITED2 as a PPAR Transcriptional Coregulator—The screening of the $3 \times 10^6$ plaques from a cDNA expression library with PPARα-MBP produced seven reproducible positive interacting clones (data not shown).2 Each clone was sequenced and compared with GenBank™. One of the interacting clones contained 437 bp that corresponds to the 3’-end of the nuclear protein CITED2 (p35srj (15)). The interaction between this partial sequence and PPARα was verified in a yeast two-hybrid assay (data not shown).

To determine whether a direct interaction occurred between CITED2 and PPARα, pull-down studies were performed. Full-length human CITED2 in the pcDNA3 mammalian expression vector was in vitro translated in the presence of [35S]methionine. Full-length rat PPARα-MBP was expressed using a bacterial expression system, the in vitro translated human CITED2 bar vector was added, and the mixture was passed over an amylose resin, washed, and eluted with excess maltose. As shown in Fig. 1, in vitro translated human CITED2 could interact with PPARα in the presence or absence of a known ligand (Wy-14,643 (Wy)), 50 μM tetradecylthioacetic acid (TTA), 10 μM prostaglandin J2 (PGJ2), or Me2SO (DMSO). Luciferase activity was determined and corrected for transfection efficiency and extraction yield.

Fig. 4. CITED2 acts as a dose-dependent coactivator of PPARα. A, COS-1 cells were transiently transfected with rPPARα fused to the GAL4-DBD and increasing amounts of CITED2. Cells were treated with 50 μM Wy-14,643 for 6 h. Luciferase activity was determined and corrected for transfection efficiency and extraction yield. Each treatment is corrected to luciferase activity with no CITED2 added (100%). Results show that CITED2 can act as a dose-dependent coactivator of PPARα in the presence or absence of ligand. *, $p < 0.05$ comparing within a chemical treatment. Values in parentheses are relative luciferase units (rlu) for the accompanying data point. B, COS-1 cells were transiently transfected with rPPARα fused to the GAL4-DBD with or without CITED2. Cells were treated with 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, or 50 μM Wy-14,643 for 6 h. Luciferase activity was determined and corrected for transfection efficiency and extraction yield. Each treatment is corrected to luciferase activity in the absence of Wy-14,643 (Me2SO (DMSO) at 100%). Graphs are representative of three independent experiments. CI, confidence interval.

RESULTS

Identification of CITED2 as a PPAR Transcriptional Coregulator—The screening of the $3 \times 10^6$ plaques from a cDNA expression library with PPARα-MBP produced seven reproducible positive interacting clones (data not shown).2 Each clone was sequenced and compared with GenBank™. One of the interacting clones contained 437 bp that corresponds to the 3’-end of the nuclear protein CITED2 (p35srj (15)). The interaction between this partial sequence and PPARα was verified in a yeast two-hybrid assay (data not shown).

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To examine the association in vivo and to map the interaction between PPARα and CITED2, mammalian two-hybrid experiments were performed. COS-1 cells were transiently transfected with full-length rat PPARα or each domain individually fused to the GAL4 DNA binding domain (pM-PPARα) and human CITED2 fused to the VP16 activation domain (VP16-CITED2). As shown in Fig. 2, an interaction between PPARα and CITED2 was observed using full-length PPARα(A–F) or just the D domain alone. If other domains were fused to the D domain, such as C or E/F, there was a noticeable decrease in

2 J. P. Gray, J. W. Davis II, T. L. Leas, C. A. Nugent, and J. P. Vanden Heuvel, manuscript submitted.

Fig. 5. CITED2 acts as a coactivator for PPARγ but not PPARα. A, HepG2 cells were transiently transfected with expression vectors for each PPAR subtype with CITED2 or empty vector control and a PPRE-driven luciferase reporter. B, COS-1 cells were transfected with GAL4-DBD-PPAR fusions for all three subtypes with and without exogenous CITED2. Transfected cells were treated for 6 h with 50 μM Wy-14,643 (Wy), 50 μM tetradecylthioacetic acid (TTA), 10 μM prostaglandin J2 (PGJ2), or Me2SO (DMSO). Luciferase activity was determined and corrected for transfection efficiency and extraction yield. Each treatment is corrected to the luciferase activity for Me2SO for each subtype. *, $p < 0.05$ comparing CITED2 bar with corresponding pcDNA3 bar. The graph is representative of three independent experiments.
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The potential coactivating ability of CITED2 was also tested with PPARβ and PPARγ using the chimeric reporter system (p-M-PPARβ and p-M-PPARγ). As shown in Fig. 5A, CITED2 acted as a strong coactivator of p-M-PPARγ basal and ligand-induced activity, while p-M-PPARβ was unaffected in the absence of exogenous ligand and was slightly repressed by CITED2 in the presence of ligand. The enhancement of p-M-PPARγ activity by CITED2 alone (MeSO-treated) was not further increased by adding the ligand prostaglandin J2. Cotransfecting HepG2 cells with a PPRE-driven reporter and pBK/PPARα, -β, or -γ resulted in a similar subtype difference in the ability of CITED2 to regulate gene expression (Fig. 5B). Overexpressing CITED2 increased the ligand-inducible PPRE-reporter activity of PPARα and PPARγ while having no effect on PPARβ. The ability of CITED2 to affect PPARγ activity is currently being explored and will not be discussed in the present report.

Effects of CITED2 on PPARα Ligand Responses in Hepatocytes—Since CITED2 is able to affect multiple transcriptions factors, including PPARα and PPARγ in cell culture, whether these proteins are coexpressed in tissues was of interest. Analysis of total RNA from 10 different mouse tissues revealed that CITED2 mRNA was ubiquitously expressed with detectable levels in all tissues tested (Fig. 6). The MuSH cell line (13) and 3T3-L1 mouse preadipocytes also expressed relatively high levels of CITED2 mRNA (data not shown). Thus, CITED2 is found in tissues that express PPARα and PPARγ.

Undifferentiated 3T3-L1 preadipocytes were transiently transfected with a construct expressing a double-stranded RNA molecule to target CITED2 for selective inhibition (RNAi). Also included in the transfection were p-M-PPARα and GAL4 reporter vectors. Subsequently the cells were treated with PPARα ligands and assayed for luciferase activity. As shown in Fig. 7, cells expressing reduced levels of CITED2 RNAi were less responsive to Wy-14,643 activation of PPARα. In addition, the basal level of PPARα activity was reduced in the presence of the CITED2 RNAi plasmid.

Due to low transfection efficiency, to examine the effects of manipulated CITED2 protein levels on endogenous gene expression, it was necessary to generate cells that harbor a stable inhibition or overexpression of CITED2. Mouse immortalized hepatocytes were stably transfected with CITED2 RNAi (CITED2–) or CITED2 cDNA (CITED2+). Control lines were also
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Effects of CITED2 Overexpression on Gene Expression in Hepatocytes—Due to the effects of CITED2 overexpression on basal gene expression and cell growth, microarray analysis was performed using a cross-hybridization between untreated empty vector control stable cells and untreated CITED2-overexpressing stable cells. The results from these arrays led to a small list of genes that were significantly affected by exogenous CITED2 (Table II and see supplemental data). Two of these genes, FOXc2 and MKP-1 were verified as being CITED2-regulated using real time PCR (Fig. 9).

DISCUSSION

Similar to other nuclear receptors, PPARα regulates gene expression by a variety of protein–protein interactions. In addition to its heterodimerization partner retinoid X receptor, PPARα associates with Hsp70 (16), Hsp90 (11, 17), liver X receptor (18), CCAAT/enhancer-binding protein α (19), and growth hormone factor-1 (20). Transcriptional coactivators and
co-repressors (collectively referred to as coregulators) are an important class of receptor-interacting protein (21). PPAR co-regulators have been identified by a variety of techniques including yeast two-hybrid assays, transient transfections, and biochemical means. Known PPARα coregulators include the coactivators PPAR-binding protein/TRAP220/DRIP205 (22), steroid receptor coactivator-1 (3), p300 (24), CBP (25), and PPARγ coactivator-1 (26) and the corepressor RIP140 (27). In the present studies we demonstrated that CITED2 acts as a PPARα coactivator capable of increasing transcriptional activity from a PPRE or from heterologous promoters. Similar to other coactivators, the CITED2 association occurred within the ligand binding domain of PPARα (D–F), and the interaction was inducible by addition of a variety of ligands.

There are two characteristics of the PPARα/CITED2 association that are dissimilar to the classic NR/coactivator interaction. First, unlike steroid receptor coactivator-1 and PPARγ coactivator-1, the NR AF-2 domain did not appear to be utilized by PPARα for CITED2 interaction. In fact, the strongest interaction occurred within the hinge region (D domain) of PPARα. Second, CITED2 was capable of acting as a ligand-independent coactivator; this is not often seen with classic coactivators.

![Regulation of gene expression by peroxisome proliferators is affected by alterations in CITED2 expression. A–E, MuSH wild type cells that were stably transfected with human CITED2 or CITED2 RNAi were treated with 50 μM Wy-14,643, 100 μM CLA, 100 μM ciprofibrate (Cipro), or MeSO (DMSO) for 6 h. Total RNA was isolated and used in real time transcription-PCR for known PPARα-regulated genes: Angpl4 (A), HIF1α (B), FOXc2 (C), MKP-1 (D), and VEGF-D (E). The MeSO level for each corresponding empty vector control was set to 100%. *, different than MeSO-treated cells within the same cell line (p < 0.05). F–J, data are identical to that presented in A–E with grouping based on treatment. *, different than control stably transfected cells within the same treatment (p < 0.05). The stable cell lines are pooled populations of transfected cells. Graphs are representative of two independent experiments.

**TABLE I**

**PCR primers**

Full-length coding sequences for genes of interest were obtained from GenBank (National Center for Biotechnology Information). Suitable real time PCR primers were designed using PrimerExpress (Applied Biosystems). Standard PCR primers (CITED2 only) were designed using PrimerSelect (DNAStar, Madison, WI). All primers are listed 5′–3′.
Table II
Genes significantly regulated by CITED2 overexpression

Gene expression microarrays were performed as detailed under "Experimental Procedures." The normalized ratios represent CITED2+/pcDNA3 stably transfected hepatocytes. Bold text indicates genes found to be linked using Pathway Assist (Version 2.01, Fig. 10).

| Common name | GenBank™ accession no. | Description | Mean | S.E. |
|-------------|------------------------|-------------|------|------|
| Oasl2       | AF068835               | 2’5’-Oligoadenylate synthetase-like 2 | 1.892 | 0.127 |
| Foxe2; Mfh1; Fkh14; MFH-1; Hffh3 | Y08222 | Mus musculus MFH-1 gene | 1.872 | 0.535 |
| Olrf69      | NM_013621              | Olfactory receptor 69 | 1.851 | 0.505 |
| 1600017K21Rik | AF192499           | RIKEN cDNA 1600017K21 gene | 1.758 | 0.457 |
| Mesp2       | U71125                 | Desmодerm posterior 2 | 1.746 | 0.567 |
| Den         | X53929                 | Decorin | 1.744 | 0.270 |
| Chm         | AF218084               | Choroideremia | 1.688 | 0.318 |
| Ifit1       | U43084                 | Interferon-induced protein with tetratricopeptide repeats 1 | 1.662 | 0.194 |
| Rock1       | U58512                 | Rho-associated coiled-coil-forming kinase 1 | 1.652 | 0.262 |
| Nr6a1       | U14666                 | Nuclear receptor subfamily 6, group A, member 1 | 1.625 | 0.220 |
| Dlk1; SCP1; pref-1 | D16847           | Mouse mRNA for stromal cell-derived protein-1, complete cds | 1.607 | 0.210 |
| Sla7a5      | AB017189               | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 | 1.598 | 0.091 |
| Spry2, SPR3 | Y09227                 | M. musculus SPR3 gene | 1.597 | 0.255 |
| Peg8        | AF038939               | Paternally expressed 3 | 1.574 | 0.271 |
| Wnt10a      | U61969                 | Wingless-related MMTV™ integration site 10a | 1.556 | 0.152 |
| Saα4        | U02554                 | Serum amyloid A 4 | 1.553 | 0.263 |
| Ifg1r       | AF06187                | Insulin-like growth factor receptor 1 | 1.540 | 0.297 |
| Zkhl1       | Z54200                 | Zinc fingers and homeoboxes protein 1 | 1.534 | 0.207 |
| Nova1       | AF232828               | Neuro-ontological ventral antigen 1 | 1.534 | 0.185 |
| Rbm14; p16; p16K; Sytip | X53129          | M. musculus p16K gene for 16-kDa protein | 1.522 | 0.219 |
| Pgc1c       | L03814                 | Neural proliferation, differentiation and control gene 1 | 1.522 | 0.080 |
| Nat2        | U35866                 | N-Acetyltransferase 2 (arylamine N-acetyltransferase) | 1.512 | 0.223 |
| Ptpn16; DUSP1; MKP-1 | X61940 | Protein-tyrosine phosphatase, non-receptor type 16 | 1.509 | 0.154 |
| Adh7        | U20257                 | Alcohol dehydrogenase 7 | 0.669 | 0.120 |
| Ltbp1       | AF022889               | Latent transforming growth factor-β-binding protein 1 | 0.566 | 0.089 |
| Idβ3        | M60523                 | Inhibitor of DNA binding 3 | 0.665 | 0.073 |
| Sort1       | A172579                | Sortilin 1 | 0.661 | 0.090 |
| Mcpr        | X50189                 | Meiotic check point regulator 1 | 0.659 | 0.099 |
| Ly78        | D37797                 | Lymphocyte antigen 78 | 0.659 | 0.094 |
| Hex1α       | U40720                 | Homeobox gene expressed in ES cells | 0.657 | 0.083 |
| Fgf; VEGFB  | X99572                 | c-Fos-induced growth factor (VEGF-b) | 0.655 | 0.094 |
| Bves        | AF124510               | Blood vessel endothelial cell substance | 0.655 | 0.098 |
| Tgm2        | M55154                 | Transglutaminase 2, C polypeptide | 0.654 | 0.107 |
| Dlk3        | U79738                 | Distal-less homeobox 3 | 0.650 | 0.091 |
| Eps15       | L21768                 | Epidermal growth factor receptor pathway substrate 15 | 0.648 | 0.101 |
| Hpiec35     | L43371                 | Hydrogen peroxide-inducible protein 53 | 0.633 | 0.071 |
| Mad3        | U23294                 | Max dimerization protein 3 | 0.632 | 0.079 |
| Uchl1       | NM_011670              | Ubiquitin carboxyl-terminal hydrolase L1 | 0.631 | 0.098 |
| Myl1r, pending | NM_021488            | Myotilin-related peptide | 0.625 | 0.114 |
| Aqp3        | NM_009701              | Aquaporin 3 | 0.619 | 0.091 |
| Krt2-18     | AF021836               | Keratin complex 2, basic, gene 18 | 0.618 | 0.131 |
| Nqo1        | U12961                 | NAD(P)H dehydrogenase, quinone 1 | 0.616 | 0.083 |
| T2          | AF072546               | Brachyury 2 | 0.613 | 0.140 |
| Zac1; PlagL1 | AF147765              | Zinc finger protein regulator of apoptosis and cell cycle arrest | 0.606 | 0.096 |
| Anxa6       | X13460                 | Annexin A6 | 0.596 | 0.080 |
| Vps45       | U66885                 | Vacuolar protein sorting 45 (yeast) | 0.587 | 0.099 |
| Prm2; Prm-2; AI528784 | X07626      | Mouse proteamine 2 gene | 0.581 | 0.104 |
| Tesc, pending | AF234783              | Tescalcin | 0.581 | 0.142 |
| Itgα2β      | NM_010575              | Integrin αβ | 0.558 | 0.144 |
| Cnn1; Cn; Cn1 | L49022               | Alternative; M. musculus h1-calponin α and β genes, complete cds | 0.550 | 0.105 |
| Il13ra2     | U65747                 | Interleukin 13 receptor, α 2 | 0.548 | 0.139 |
| Nr113       | AF009327               | Nuclear receptor subfamily 1, group I, member 3 | 0.518 | 0.126 |
| Igfph2      | X81580                 | Insulin-like growth factor-binding protein 2 | 0.511 | 0.089 |
| Art1        | U3510                  | ADP-ribosyltransferase 1 | 0.504 | 0.135 |
| Rangmr, pending | AF124510             | RAN guanine nucleotide release factor 1 | 0.485 | 0.126 |
| B3gal3t3    | AF029792               | Glycosyltransferase, βGalT-III; M. musculus UDP-Gal:βGlcNAc β1,3-galactosyltransferase-III (β3GT3) gene, complete cds | 0.470 | 0.126 |
| Adey8       | U58021                 | Adenylylate cyclase 8 | 0.428 | 0.131 |
| Mglap       | D00613                 | Matrix γ-carboxyglutamate (gla) protein | 0.366 | 0.125 |
| Krt2-8      | X12789                 | Keratin complex 2, basic, gene 8 | 0.353 | 0.089 |

*Murine mammary tumor virus.

Embryonic stem.

Coding sequence.
Increasing amounts of CITED2 increased reporter activity in the absence of exogenous ligand from a PPRE- or a GAL4-driven reporter. CITED2 is dynamically regulated, being induced by hypoxia (15), shear stress (28), cytokines, and serum (29) and has a distinct pattern of expression during development (30). Thus, under conditions of high CITED2 expression, there may also be increased PPARα activity irrespective of the addition of exogenous ligand. This is important due to the role PPARα plays in lipid metabolism as well as cell cycle regulation.

The biological role of CITED2 has become an area of interest in regard to embryonic development and cancer. Disruption of the gene encoding CITED2 is embryonic lethal due to defects in the development of heart and neural tube (8), although treatment with folic acid significantly reduced the neural tube defects in the Cited2−/− embryos (31). Proliferation of Cited2−/− mouse embryonic fibroblasts ceases prematurely with a reduction in growth fraction, senescent cellular morphology, and increased expression of the cell proliferation inhibitors p16(INK4a), p19(ARF), and p15(INK4b) (10). Overexpression of CITED2 in Rat1 cells resulted in loss of cell contact inhibition, anchorage-independent growth in soft agar, and tumor

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**Fig. 10. Analysis of altered gene expression in the CITED2+ cells.** Genes that were significantly regulated in gene expression microarrays (CITED2+/pcDNA3, Table II) were examined using Pathway Assist (Version 2.01). The pathway was built by looking for common regulators of the genes shown in Table II, and the predominant cluster is depicted. The lines and arrows depict observations on regulation of gene expression (+, increased expression; −, decreased expression) from the literature. The effects of CITED2 overexpression on the amount of mRNA in the microarray experiments are shown (black, significantly increased; gray, significantly decreased; white, no significant effect observed). Following creation of this cluster, the proteins in the gradient filled ovals were included (PPARα, PPARγ, Angpt14, and HIF1α), and the connections were determined by Pathway Assist or by manually adding (PPARα and CITED2 interaction). EGF, epidermal growth factor; TGF, transforming growth factor; DCN, decorin; AQP5, aquaporin 5; TNF, tumor necrosis factor; IFG1R, insulin-like growth factor I receptor; IL2, interleukin 2; IGFBP2, insulin-like growth factor-binding protein 2; IFN, interferon; LTBP1, latent transforming growth factor-β-binding protein 1; EPS15, epidermal growth factor receptor pathway substrate 15; TGM2, transglutaminase 2; UCHL1, ubiquitin carboxyl-terminal hydrolase L1; FIGF, c-fos-induced growth factor; GHRL, growth hormone receptor, long form; IL13R, interleukin 13 receptor; GHI, growth hormone 1; ADCY8, adenylate cyclase 8; TSA, trichostatin A; MGP, matrix γ-carboxyglutamate protein.
formation in nude mice (29). In the present studies, overexpression of CITED2 led to an increase in cell growth rates, while the use of RNAi to repress this protein had little effect. The lack of effect of the knock-down studies may represent a difference in cell type (fibroblast versus hepatocytes) or the fact that incomplete repression of CITED2 levels was produced by RNAi techniques. Nonetheless it has now been demonstrated that overexpression of CITED2 can increase cell proliferation of hepatocytes as well as fibroblasts.

The role of CITED2 as a coregulator was originally described for HIF1α where CITED2 inhibited HIF1α transactivation by blocking its interaction with CBP/p300 (15). In addition to modulating the basic helix-loop-helix, Per-Arnt-Sim family member HIF1α, CITED2 is a coactivator of basic-helix-loop-helix DNA-binding protein AP2 (33). The present studies showed that CITED2 can affect a third class of transcription factors, nuclear receptors, and that CITED2 acts as a coregulator of both PPARα and PPARγ. Virtually all of the of the CITED2 in the cell is associated with the CBP/p300 complex (15). Thus, CITED2 may affect the ability of a subset of CBP/p300-associated transcription factors to regulate gene expression in either an inhibitory (HIF1α) or stimulatory (PPAR and AP2) manner. Since CBP/p300 is an essential component of many transcriptional complexes, there is potential for CITED2 to affect the activity of a wide range of proteins. Interestingly our work has shown some specificity in that PPAR-β activity was not affected by CITED2 overexpression even though CBP/p300 is a coactivator of this receptor (34).

Since CITED2 mRNA is increased markedly by hypoxia or deferoxamine, it has been suggested to operate in a negative feedback loop with HIF1α (15). HIF1α transcribes CITED2 during hypoxia, and the CITED2 that accumulates during restoration of normal oxygen homeostasis will inhibit HIF1α transactivation and, in turn, its own production (35). The increase in HIF1α target gene products including VEGF-D (8) in CITED2−/− fibroblasts is further evidence for this model (35). Increasingly there are connections being made between PPARs, HIF1α, and angiogenesis. Hypoxia via HIF1α inhibits the expression of PPARs (36) and decreases PPARα/retinoid X receptor binding to its response element (37). During adipocyte dedifferentiation PPARγ is decreased, while HIF1α mRNA expression increases (38). PPARγ ligands in particular have received considerable attention as angiogenesis inhibitors (23). As discussed below, several angiogenesis-related genes are regulated by PPARα ligands. The CITED2/p300 complex may increase the ability of PPAR to decrease angiogenesis (via regulation of key target genes) while also inhibiting HIF1α and its ability to stimulate this process.

Several cell lines were generated to examine the role of CITED2 in endogenous gene expression and in the ability of PPARα ligands to affect certain transcripts in hepatocytes. Previous work has shown that the model hepatocyte system (MuSH) was appropriate for examination of cell proliferation and PPARα-dependent gene expression (13). The genes chosen for examination included those potentially involved in angiogenesis and previously shown to be PPAR-regulated (Angptl4 and MKP-1) or CITED2-regulated from previous studies (VEGF-D and HIF1α) or from the current microarray experiments (FOXc2). The present data confirms that Angptl4 and MKP-1 are regulated by PPAR ligands; FOXc2 can also be added to this list. In most instances CLA exerted a more dramatic effect on gene expression perhaps due to the fact that it is also a ligand for PPARγ (32). Although seen in the microarray experiments, using real time PCR we were unable to confirm VEGF-D as being affected solely by CITED2 expression in the hepatocyte model system.

The determination of an endogenous PPARα-regulated gene that uses CITED2 as a coactivator remains elusive. Evidence for such a gene would be an increase in ligand inducibility in the CITED2+ cells with an absence (or significant diminution) of this regulation in the CITED2− hepatocytes. Perhaps the gene that comes closest to this pattern is MKP-1 (Fig. 9f), although FOXc2 regulation by CLA and ciprofibrate may utilize CITED2 for coactivation. Interestingly the desired trend of activity in the four cell lines was observed with cell proliferation (Fig. 8b). This suggests that certain growth-regulatory genes may utilize a PPARα/CITED2-containing transcription complex.

An examination of the pathways affected by CITED2 supplementation revealed a small group of genes that are coordinately regulated (Fig. 10). Initially genes found to be regulated in the microarray experiments were utilized to cluster the data, and subsequently the key proteins in the present study (PPARα and -γ, HIF1α, and Angptl4) were included. A central signaling molecule in this pathway is tumor necrosis factor, which has been implicated in the regulation of five of the genes found to be affected by CITED2 overexpression, and it in turn affects the activity of PPAR and HIF1α. In addition to FOXC2 and MKP-1, tumor necrosis factor has previously been reported to increase the expression of WNT10A and decorin while repressing aquaporin. Epidermal growth factor, interleukin 2, and transforming growth factor-β also figure prominently in this pathway. Thus, it is attractive to speculate that CITED2 affects, in addition to PPAR, a common factor in the tumor necrosis factor, epidermal growth factor, and transforming growth factor-β signaling cascades. PPARα is a regulator of MKP-1 and Angptl4, and the present studies suggest it also affects FOXC2 mRNA accumulation. HIF1α regulates Angptl4 and is negatively regulated by CITED2. It is evident from examining Fig. 10 that a reason for the difficulty in finding an obvious endogenous gene being regulated by a PPARα-CITED2 complex is the multiple, competing pathways that are affecting this cluster of proteins.

The results described here show that the CBP/p300-interacting protein CITED2 is a coactivator of PPARα-mediated transcriptional activity. The coactivating ability of CITED2 is present over a wide range of known peroxisome proliferators, and this protein is also a basal coactivator for PPARα. This work has also shown that overexpression of CITED2 affects cell proliferation in hepatocytes, a phenomenon previously observed in fibroblasts. Thus, there may be a significant physiological role for CITED2 protein in the transcriptional regulation of PPARα target genes in the liver in particular for those involved in the cell cycle.

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