Characterization of Novel Peroxisome Proliferator-activated Receptor γ Coactivator-1α (PGC-1α) Isoform in Human Liver

Thomas K. Felder, Selma M. Soyal, Hannes Oberkofler, Penelope Hahne, Simon Auer, Richard Weiss, Gabriele Gadermaier, Karl Miller, Franz Krempler, Harald Estebauer, and Wolfgang Patsch

From the Department of Laboratory Medicine, Paracelsus Medical University, 5020 Salzburg, Department of Molecular Biology, University of Salzburg, 5020 Salzburg, Departments of Surgery and Internal Medicine, Krankenhaus Hallein, 5400 Hallein, and Department of Laboratory Medicine, Medical University Vienna, 1090 Vienna, Austria

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a transcriptional coactivator that contributes to the regulation of numerous transcriptional programs including the hepatic response to fasting. Mechanisms at transcriptional and post-transcriptional levels allow PGC-1α to support distinct biological pathways. Here we describe a novel human liver-specific PGC-1α transcript that results from alternative promoter usage and is induced by FOXO1 as well as glucocorticoids and cAMP-response element-binding protein signaling but is not present in other mammals. Hepatic tissue levels of novel and wild-type transcripts were similar but were only moderately associated (p < 0.003). Novel mRNA levels were associated with a polymorphism located in its promoter region, whereas wild-type transcript levels were not. Furthermore, hepatic PCK1 mRNA levels exhibited stronger associations with the novel than with the wild-type transcript levels. Except for a deletion of 127 amino acids at the N terminus, the protein, termed L-PGC-1α, is identical to PGC-1α. L-PGC-1α was localized in the nucleus and showed coactivation properties that overlap with those of PGC-1α. Collectively, our data support a role of L-PGC-1α in gluconeogenesis, but functional differences predicted from the altered structure suggest that L-PGC-1α may have arisen to adapt PGC-1α to more complex metabolic pathways in humans.

PGC-1α (PPARGC1A) influences transcription in an exceptional variety of pathological pathways including adaptive thermogenesis (1), mitochondrial biogenesis (2), skeletal muscle fiber determination and neuromuscular junction formation (3, 4), angiogenesis (5, 6), hepatic gluconeogenesis (7–9), fatty acid β-oxidation (10), regulation of clock genes (11), and protection of neural cells from reactive oxygen species (12, 13). Recent reviews describe the numerous functions of this fascinating protein (14–17).

Several levels of regulation have been implicated to explain the diverse roles of PGC-1α and its interactions with distinct transcription factors. For some pathways, expression levels of PGC-1α and transcription factors coactivated by PGC-1α are crucial (18). In addition, various signaling pathways target PGC-1α at the post-translational level. Such modifications detailed recently (19) alter the stability of PGC-1α and/or direct interactions with specific factors, thereby enhancing distinct transcriptional programs.

Alternative splicing and/or transcription initiation, resulting in gain or deletion of interacting domains or signaling targets, represents another mode of regulation (15). Several PGC-1α isoforms have been reported in animal models (20, 21). A short PGC-1α isoform was shown to be coexpressed with wild-type PGC-1α in mouse tissues and in human heart (22). The alternatively spliced mRNA is translated into a truncated protein, termed NT-PGC-1α, that retains the N-terminal transactivation and nuclear receptor interaction domains and is functionally active.

Knowledge about PGC-1α expression and regulation in human tissues is limited. However, such information is important because PGC-1α has been implicated in human disorders as diverse as type 2 diabetes mellitus (23–25) and Huntington disease (12, 26–28). Here we report the sequence, subcellular localization, and some relevant functional properties of a novel PGC-1α isoform in human liver, termed L-PGC-1α.

EXPERIMENTAL PROCEDURES

Study Subjects—The study included 68 obese but otherwise healthy female patients. Participants were included if they had fasting plasma glucose levels <7.0 mmol/liter, C-reactive protein-binding protein; L, liver; NT, novel truncated; PPAR, peroxisome proliferator-activated receptor; RLM, liver X receptor α; MCAD, medium-chain fatty acyl-CoA dehydrogenase; mthHNF4α, mutant HNF4α; G6P, glucose-6-phosphatase.
tein levels <30 mg/liter, no history of diabetes or use of lipid-lowering medication, and no weight changes >3% during the previous 2 months. They underwent a surgical weight-reducing procedure including a liver biopsy after an overnight fast. Tissue samples were collected in RNAlater (Ambion). Study participants provided informed consent, and study protocols were approved by the local ethics committee.

**Plasmids**—Expression plasmids pLXRα, pFOXO1, pFOXO2, pSREBP-1c, and pPGC-1α as well as the promoter-luciferase reporter construct pSREBP-1c-LUC were described earlier (25, 29–33). Plasmids pHNF4α and pL-PGC-1α and the enhanced GFP in-frame fusion constructs pL-PGC-1α-GFP and pPGC-1α-GFP were cloned into pcDNA6-V5/His A (Invitrogen) or pEGF-P-N1 (Clontech), respectively. pcDNA6 was also used for generating human expression plasmids pPPARα and pPPARγ. The human promoter-luciferase reporter vectors pc36-Prom-Luc, pMCAD-Prom-Luc, pPCK1-Prom-Luc, pPGC-1α-Prom(2647)-Luc, and pL-PGC-1α-Prom(3518)-Luc were generated in the pGL4 backbone (Promega). Two truncations of the promoter region were created from pL-PGC-1α-Prom(3518)-Luc, namely pL-PGC-1α-Prom(1158)-Luc and pL-PGC-1α-Prom(542)-Luc. The predicted binding sites for forkhead box 01A (FOXO1), CREB1 and NRC31 (glucocorticoid receptor) of the latter plasmid as well as the AF-2 domain (L374A and L375A) in pHNF4α and pL-PGC-1α-Halo were altered using the QuikChange site-directed mutagenesis kit (Stratagene). L-PGC-1α was cloned into pCI TPA Art Tet, kindly provided by A. Hartl (34), for DNA vaccination. Plasmids for the ribonuclease protection assay (RPA) and Northern probe generation by *in vitro* transcription were obtained by T/A cloning of adequate PCR amplicons into the pGEM-T Easy Vector System (Promega). The C-terminal Halo-tagged versions pL-PGC-1α-Halo, pPGC-1α-Halo, pHNF4α-Halo, and pmutHNF4α-Halo used for chromatin isolation and pulldown analyses were generated in the pHTC HaloTag® CMV-neo Vector (Promega). All plasmids were generated by standard molecular biology cloning techniques and verified by DNA sequencing using the ABI 3500 genetic analyzer (Applied Biosystems). Primers used for the generation of the respective amplicons are given in supplemental Table S1.

**RNA Analyses**—Total RNA was isolated from Sprague-Dawley rat liver, human liver, and HepG2 cells using RNeasy Mini or Midi kits (Qiagen) and digested with DNase I (Promega). Poly(A+)-selected RNA was ascertained by electrophoresis in denaturing formaldehyde-agarose gels. We purchased the FirstChoice Human Total RNA Survey Panel and Swiss Webster mouse liver RNA (Applied Biosystems/Ambion) and hepatic total RNA from dog and rhesus monkey (Biochain). Rat liver RNA (Applied Biosystems/Ambion) and hepatic total RNA from dog and rhesus monkey (Biochain) was used for RLM-RACE analyses according to the manufacturer’s instructions. Briefly, 1 μg of total human liver RNA was treated with calf intestine alkaline phosphatase to remove free 5’-phosphates, leaving the 5’-cap structure of full-length mRNA intact. The cap was removed by incubation with tobacco acid pyrophosphatase prior to ligation.
from exon 10/11 containing transcripts for estimation of full-length PGC-1α transcripts. We verified the accuracy of the customized assay by sequencing the cloned amplicons in three individuals. To directly compare measurements of PGC-1α transcript regions, gene segments containing the sequences targeted by the respective TaqMan assays were used to construct standard curves. Data are presented in arbitrary units relative to RPLP0 mRNA. For typing rs12500214, we used a TaqMan genotyping assay (C_11325181_10, Applied Biosystems).

**Cell Culture and Transfection Experiments**—Human hepatoma HepG2 cells were grown as recommended by the supplier (American Type Culture Collection). HepG2 cells cultured in 24-well dishes were transfected using Lipofectamine 2000 reagent (Invitrogen) as described (25). We used 0.2 μg of reporter plasmids, 0.5 μg of expression plasmids, and 20 ng of pRL-TK plasmid (Promega) as transfection control per well. Cells were collected 24 h after transfection, and firefly and Renilla luciferase activities were measured with a GloMax Multi Detection System luminometer (Promega) using the Dual-Luciferase System luminometer (Promega) as described (25). Results are representative of two experiments, each performed in quadruplicate, and are given as means ± S.D. Dexamethasone, 8-bromoadenosine 3’5’-cyclic monophosphate (8-Br-cAMP), WY14643, troglitazone, 22(R)-hydroxycholesterol, and 9-cis-retinoic acid were obtained from Sigma-Aldrich and used at the concentrations indicated.

**Fluorescence Microscopy**—A Zeiss Axioskop microscope equipped with an oil immersion ×100 objective lens and a video camera was used for fluorescence and differential interference contrast microscopy. HepG2 cells were transfected with plasmid pL-PGC-1α-GFP or pPGC-1α-GFP. Visualization of nuclei and mitochondria in living cells was performed with 4’,6’-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) DNA staining and MitoTracker® Red CMXRos (Invitrogen, Molecular Probes), respectively.

**In Vitro Transcription/Translation**—Plasmids pPGC-1α and pL-PGC-1α and the TNT® Quick Coupled Transcription/Translation System (Promega) were used for *in vitro* synthesis of wild-type and L-PGC-1α. Briefly, 1 μg of circular plasmid DNA was added to TNT lysate, reaction buffer, RNA polymerase, ribonuclease inhibitor, and non-radioactive amino acids and incubated at 30 °C for 90 min. Samples were denatured in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromphenol blue) at 95 °C for 5 min, cooled on ice, and subjected to electrophoresis in SDS-polyacrylamide gels.

**Immunoblotting**—Human brain tissue protein extract (Millipore, catalogue number CL302) and liver nuclear extract (Active Motif, catalogue number 36042) were used for immunoblotting. Nuclear and cytoplasmic extracts from HepG2 cells grown to confluence in 70-cm² flasks were prepared using the NE-PER™ kit (Pierce Thermo Fisher Scientific). Protein concentrations were determined using the BCA Protein Assay (Pierce Thermo Fisher Scientific). Equal amounts of nuclear extracts per lane (15 μg) were subjected to SDS-polyacrylamide gel electrophoresis followed by electrotransfer to an Amersham Biosciences Hybond™-P PVDF transfer membrane (GE Healthcare) as described (32). After blocking with 1× TBS containing 5% (w/v) nonfat dry milk for 1 h at 22 °C, membranes were incubated overnight at 4 °C with monoclonal antibody PGC-1α (3G6 rabbit mAb, Cell Signaling Technology), polyclonal IgG PGC-1α (K-15, sc-5816 antiserum, Santa Cruz Biotechnology), or antisera obtained by DNA vaccination. After washing and incubation with the secondary anti-rabbit HRP-linked (Cell Signaling Technology) or anti-mouse IgG HRP-linked antibody (Pierce Thermo Fisher Scientific) for 1 h at 22 °C, blots were exposed to SuperSignal West Dura substrate (Pierce Thermo Fisher Scientific), and chemiluminescence signals were recorded using the Kodak Imaging Station 2000 MM.

**Chromatin Immunoprecipitation (ChiP) Assays**—We used the HaloChip System (Promega), which provides a robust alternative to the standard ChiP method by capturing protein-DNA complexes from mammalian cells without the need for antibodies. The HaloTag protein is fused to the protein of interest via cloning into a HaloTag vector and mediates a covalent interaction with a resin-based ligand. C-terminally Halo-tagged versions of PGC-1α, PGC-1α, or HNF4α or the empty HaloTag vector was transiently expressed in HEK293 or HepG2 cells for 24 h and subsequently treated for 10 min with formaldehyde (1%, v/v) to induce covalent protein-DNA cross-links. Cross-linking was quenched by the addition of glycine to a final concentration of 125 mM. Cells were pelleted in PBS and frozen at ~70 °C for 10 min prior to lysis by mechanical disruption using 25 strokes of a Dounce homogenizer. Lysates were sonicated using a Branson sonicator (13 cycles of 15 s each with 1 min of cooling on ice between cycles) to shear chromatin to a median fragment size of ~500 bp. Cross-linked complexes containing HaloTag proteins were captured using HaloLink resin according to the manufacturer’s recommendations. After stringent washing to remove nonspecific proteins and DNA, captured DNA fragments were released by heating for 6 h at 65 °C and purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Purified DNA was subjected to PCR amplification using primers spanning the HNF4α binding site in the PCK1 promoter (39). Cells transfected with the empty pHTC HaloTag vector were used as a negative control. As an additional control, an aliquot of the L-PGC-1α-Halo lysate was incubated with blocking ligands, preventing the interaction of the Halo-tagged protein with the HaloLink resin.

**Pulldown Analyses**—We used the HaloTag Mammalian Pull-Down System (Promega) according to the manufacturer’s instructions. HEK293 cells (2 × 10⁷) were transiently transfected with C-terminally Halo-tagged versions of L-PGC-1α or HNF4α as baits to capture interacting proteins. Cells expressing the HaloTag control vector served as a negative control. Cells were lysed 36 h after transfection, and nuclear extracts were prepared as described (36). HaloTag fusion proteins along with their interacting proteins were captured using the HaloLink resin and washed gently. Interacting proteins were eluted from the resin with SDS elution buffer and subjected to SDS-PAGE followed by electroblotting. Blots were probed with mouse monoclonal HNF4α antibody (sc-101059, Santa Cruz Biotechnology) and anti-mouse IgG HRP-linked antibody (Pierce Thermo Fisher Scientific) as secondary antibody. Monoclonal histone H3 antibody (3H1 rabbit mAb, Cell Signal-
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ing Technology) was used as a loading control of nuclear extracts.

**DNA Vaccination**—Female BALB/c mice (6–8 weeks of age; Charles River, Sulzfeld, Germany) were immunized via gene gun three times at weekly intervals. L-PGC-1α, cloned into pcDNA3.1 (Invitrogen), was precipitated onto gold beads (1.6–μm diameter) with CaCl2 in the presence of spermidine at a loading rate of 2 μg/mg of gold. Mice received a total of 2 μg of DNA per immunization, divided between two non-overlapping areas, on the shaved abdomen at a helium pressure of 400 p.s.i. Blood samples were taken before and 2 weeks after the third immunization. Mice were boosted by gene gun 11 weeks after the third immunization and exsanguinated 1 week later (34, 40). All animal experiments were approved by the local animal committee.

**Computational and Statistical Analyses**—Linear regression analyses were performed using log-transformed hepatic mRNA levels. Effects of genotypes associated with rs15200214 on L-PGC-1α transcript levels were ascertained by analysis of variance adjusted for age and body mass index. Transactivation assays were analyzed by analysis of variance. Allele frequencies were estimated by gene counting. Agreement with Hardy-Weinberg expectations was tested using a χ² goodness-of-fit test. To identify transcription initiation sites and evolutionarily conserved regions we used PROMO (41) and rVISTA (42).

**Accession Number**—The sequence of the transcript encoding L-PGC-1α has been deposited in GenBank™ under accession number HQ695733.

**RESULTS**

**Identification and Characterization of Novel PGC-1α Transcript in Human Liver and HepG2 Cells**—Initially, we used cDNA from various tissues to perform PCR yielding amplicons evenly spread over the entire human PGC-1α mRNA. Marked reductions of PCR products spanning exons 2–4 relative to other exon-spanning amplicons of comparable length were observed in cDNA from liver but not from skeletal muscle or kidney (data not shown). To identify alternative transcripts, we used RLM-RACE, a technique that restricts cDNA amplification to 5′-capped mRNAs. RACE-ready cDNA prepared from liver biopsies of three patients served as templates for 5′-RLM-RACE from exon 4. Two distinct amplification products were observed in all three subjects (Fig. 1A). Sequencing showed that the larger 847-bp amplicon contained an alternative exon 1, termed exon 1L, located within intron 2, that was spliced to exon 3 followed by exon 4. The smaller 637-bp amplicon contained the reported wild-type sequence. Similar results were obtained with RACE-ready human liver cDNA (Ambion) and HepG2 cDNA (data not shown).

To define the start site(s) of the novel transcript, we performed 5′-RLM-RACE with a nested reverse primer located in exon 1L and observed two amplicons in liver biopsies of four subjects but no amplicon in brain mRNA (Fig. 1B). Sequencing indicated that the larger fragments differed from the shorter fragments by a 5′-extension of 41 bp (supplemental Fig. S1). Interestingly, the relative intensities of the two bands varied among individuals. As NCBI dbEST entry BX105309 showed another exon upstream of exon 1L in human testis, we performed PCR with primers located in exon 1L and the putative upstream exon but did not detect such an exon in liver. Moreover, in *silico* analyses predicted two transcriptional initiation sites in exon 1L with scores of 0.86 and 0.85 that perfectly matched the sites obtained by RLM-RACE. Hence, the novel liver transcript is generated by variable utilization of two transcription start sites in exon 1L. As described previously (37), wild-type transcripts in liver and brain were also initiated at two adjacent sites (Fig. 1B).

To confirm the expression of exon 1L in human liver and HepG2 cells, we performed RPAs using a probe spanning the exon 1L/exon 3 junction. Fragments predicted from RACE studies were protected in liver and HepG2 cells but not in skeletal muscle or kidney (Fig. 1C). Furthermore, abundance levels of transcripts with or without exon 1L were comparable. Because significant amounts of 64-nucleotide fragments protected only if exon 1L was spliced to sequences other than the acceptor site of exon 3 were not detected, exon 1L was exclusively spliced to exon 3. This conclusion was supported by 3′-RACE studies (data not shown). The location of exon 1L in the genomic PGC-1α context is displayed in Fig. 1D.

Two RPA probes extending from exon 1L or exon 2 to exon 7 were used for further characterization of hepatic PGC-1α transcripts. Liver and HepG2 RNA contained fully protected fragments with both probes (supplemental Fig. S2, A and B), but transcripts harboring exon 1L were not detected in brain RNA. To determine whether exon 1L-initiated mRNA was subject to alternative splicing similar to transcripts encoding NT-PGC-1α, we performed semi-quantitative RT-PCR and sequenced amplicons. Using liver and HepG2 mRNA as templates, we observed a minor transcript population initiated at exon 1L that was alternatively spliced and contained the intron 6 insertion (supplemental Fig. S2C).

Next, we determined the full sequence of exon 1L-initiated transcripts. Exon 1L and exon 13 primers produced amplicons specific to HepG2 or liver cDNA compared with control amplicons amplified with exon 1 and exon 13 primers and obtained in liver, skeletal muscle, and HepG2 cDNA (Fig. 1E). Sequencing of amplicons verified the exon 1L/exon 3 junction and the regular order of exons 3–13. Northern blots using mRNA and a cRNA probe hybridizing to exon 1L revealed two transcripts of ~6.4 and ~5.3 kb in liver and HepG2 cells but not in kidney or skeletal muscle (Fig. 1F). Using a probe spanning exons 2–7, transcripts of comparable sizes were observed in all mRNAs analyzed (Fig. 1G). As suggested previously (37), the size difference of 1.1 kb between these mRNA species most likely reflects usage of alternative poly(A) signals in exon 13. Thus, liver and HepG2 cell transcripts initiated in exon 1L are similar in size to wild-type transcripts present in other tissues. Hence, only probes targeting the distinct 5′-regions discriminate the two types of transcripts. In liver, HepG2 cells, and kidney and to a lesser extent in skeletal muscle, abundant small transcripts of ~0.8 kb were observed with the exon 2–7 but not the exon 1L probe. Similar data have been reported in rats (43). Because of the more effective transfer of short mRNA by diffusion blotting, their levels are probably greatly overestimated in comparison with full-length mRNA.
Novel Isoform Is Highly Enriched in Human Liver but Not Detectable in Livers of Mice or Higher Mammals—To determine the tissue-specific expression of exon 1L, we quantified exon 1L- and exon 1-containing transcripts in 18 human tissues (Fig. 2A). Exon 1- and 2-containing mRNA was expressed in most tissues but mainly in brain, kidney, liver, skeletal muscle, and thyroid. In contrast, exon 1L-containing transcripts were strongly expressed in liver and to a lesser extent in testis. We therefore termed the novel transcript \( \text{L-PGC-1} \). As observed with RPAs, the abundance levels, corrected for amplification efficiency, of wild-type and exon 1L-containing transcripts were comparable in human liver.
PGC-1α mRNA is highly conserved among mammals, and exon 1L is located within an evolutionarily conserved region. We therefore ascertained whether exon 1L homologues are expressed in livers of mammals. Using several primer pairs targeting the conserved exon 1L region and exon 3 of the mouse gene, we failed to detect transcripts in liver cDNA of overnight fasted mice. In contrast, transcripts spanning exons 1–3 were readily amplified from the same cDNA. In addition, a probe complementary to murine exons 2 and 3 protected fragments of wild-type transcripts as expected (Fig. 2B). However, 92 nucleotide-containing fragments indicative of splicing of another exon (such as exon 1L) to exon 3 were not observed. RT-PCRs using hepatic RNA from rat, dog, and rhesus monkey and primers located in fully conserved regions of exon 1L and exon 3 produced no amplicons, whereas a product was readily obtained in human liver. The adequacy of RNAs was verified in assays with universal primers located in exons 11 and 13 as amplicons were obtained in all species (Fig. 2C).

*In Vitro Transcription/Translation, Immunoblotting, and Immunocytochemistry—*Splicing of exon 1L to exon 3 eliminates the start ATG codon of PGC-1α located in exon 1. The first ATG codon, producing an open reading frame, is shifted into exon 3, resulting in deletion of the first 127 amino acids. To test whether such a shorter isoform is translated, we performed
in vitro coupled transcription/translation reactions with PGC-1α and L-PGC-1α. Both PGC-1α and L-PGC-1α (~91 and 77 kDa, respectively) were identified by immunoblotting using an antibody directed against C-terminal epitopes of PGC-1α (Fig. 3A). The same antibody recognized two proteins of sizes attributed to potential post-translationally modified PGC-1α and L-PGC-1α in HepG2 nuclear extracts, whereas a monoclonal antibody against an N-terminal epitope detected PGC-1α and NT-PGC-1α but not L-PGC-1α (Fig. 3, B and C). Antiserum produced by vaccination with DNA encoding L-PGC-1α clearly detected a band (not observed with preimmune serum) of the predicted L-PGC-1α size in nuclear extracts of HepG2 cells and liver, whereas faint bands corresponding to the size of the wild-type protein were visualized (Fig. 3, D and E). A comparison of functional domains present in PGC-1α, L-PGC-1α, and NT-PGC-1α is shown in Fig. 3F.

To ascertain the subcellular localization of L-PGC-1α, which contains a nuclear localization signal, we transiently expressed wild-type and L-PGC-1α, both C-terminally tagged with in-frame enhanced GFP, in HepG2 cells. We observed colocalization of both wild-type and L-PGC-1α with DAPI staining, indicating nuclear localization of the new isoform (Fig. 4).

The 5′-Sequence of L-PGC-1α Supports Its Transcription—To substantiate the accuracy of transcription initiation sites in exon 1L, we cloned a ~3.2-kb upstream fragment into a reporter vector and observed, in comparison with control, a 4-fold increase of transcriptional activity in transient transfections of HepG2 cells (Fig. 5A). In silico analysis predicted a
FOXO1 binding site in the putative promoter. Indeed, cotransfection of HepG2 cells with FOXO1 expression plasmids markedly induced reporter gene activity, and two truncated promoter-reporter constructs retained FOXO1 inducibility (Fig. 5A). Mutagenesis of the predicted FOXO1 core sequence in the 0.542-kb promoter construct reduced its activation by FOXO1 nearly to the control level (Fig. 5, B and C). Thus, the sequence immediately upstream of exon 1L is a functional promoter that is activated by FOXO1 in vitro. Moreover, FOXO1 and L-PGC-1α mRNA levels in livers of obese subjects are strongly correlated (Fig. 5D).

Exon 1L along with its 5'-region (harboring rs12500214) is located in a haplotype block distinct from haplotype blocks comprising the wild-type promoter or the sequence downstream of exon 2.3 We typed rs12500214 in 68 obese female subjects. Genotypes associated with rs12500214 fulfilled Hardy-Weinberg expectations and were associated with L-PGC-1α mRNA levels (Fig. 5E). Conversely, no effects of rs12500214 on full-length PGC-1α mRNA were noted (1.73 ± 2.44, 2.11 ± 1.96, and 0.60 ± 0.58 arbitrary units for genotypes GG, GA, and AA, respectively).

Glucocorticoids as well as glucagon-PKA signaling are enhanced in the fasting state, and both signaling pathways increase hepatic PGC-1α expression (9). We therefore investigated whether hepatic L-PGC-1α mRNA is also induced by these signaling cascades. HepG2 cells treated with dexamethasone showed comparable increases in L-PGC-1α and PGC-1α transcripts and larger increases in the mRNA levels of the PGC-1α targets PCK1 and G6P. At the doses used, even stronger effects on L-PGC-1α, PGC-1α, PCK1, and G6P mRNA levels were observed with the cAMP analog 8-Br-cAMP (Fig. 6A). Moreover, strong associations of L-PGC-1α and PCK1 transcript levels were noted in liver biopsies, providing an in vivo correlate for a role of L-PGC-1α in PCK1 mRNA expression (Fig. 6B). Furthermore, PGC-1α transcript levels displayed only modest associations with L-PGC-1α (r = 0.3756, p = 0.003) and PCK1 transcript levels (r = 0.3649, p = 0.005). To identify the respective cis-regulatory sites in the L-PGC-1α promoter, we transfected reporter constructs driven by L-PGC-1α promoters of different lengths into HepG2 cells. Again, treatment of cells with 8-Br-cAMP induced L-PGC-1α promoter activities more than dexamethasone. Furthermore, the stimulatory

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effects of both dexamethasone and 8-Br-cAMP increased with the extent of promoter truncations (Fig. 6C). In silico analyses suggested putative binding sites for CREB and glucocorticoid receptors (glucocorticoid response element) at position −11002 to −368 and −57 to −36, respectively. Indeed, mutagenesis of the CREB binding site and the glucocorticoid response element in the 0.542-kb promoter construct abrogated its activation by the respective treatment (Fig. 6D).

To identify potential transcriptional pathways with distinct influences on hepatic L-PGC-1α and PGC-1α promoter activities, HepG2 cells were cotransfected with equimolar amounts of L-PGC-1α and PGC-1α promoter-reporter constructs and expression plasmids encoding various transcription factors known to be active in liver (Fig. 6E). PPARγ and nuclear active SREBP-1c had no stimulatory effect on either reporter construct, whereas PPARα enhanced transcription only from the L-PGC-1α promoter. HNF4α and LXRα activated both reporter constructs. Compared with the PGC-1α promoter, the L-PGC-1α promoter was more strongly trans-activated by FOXO1, whereas the opposite effect was observed for FOXA2.

L-PGC-1α and PGC-1α Have Overlapping Coactivation Properties—To delineate potential functional differences between PGC-1α and L-PGC-1α in human systems, we compared their coactivation properties by cotransfection of HepG2 cells with L-PGC-1α and PGC-1α expression vectors along with reporter constructs driven by promoters of various nuclear receptors (NRs). As PGC-1α was originally identified as a coactivator of PPARγ, we examined the ability of L-PGC-1α to coactivate the CD36 promoter containing a bona fide PPARγ response element with activity in liver (44). L-PGC-1α and PGC-1α showed comparable coactivation potencies when equimolar amounts of expression plasmids were used (Fig. 7A). Coactivation of PPARα and HNF4α, demonstrated previously for PGC-1α (45, 46), was determined with MCAD and PCK1 promoters, respectively. Again, comparable activities for L-PGC-1α and PGC-1α were observed (Fig. 7, B and C). However, only PGC-1α coactivated LXRα at the SREBP-1c gene promoter (Fig. 7D).

L-PGC-1α physically interacts with HNF4α and is recruited to PCK1 promoter—As our in vitro reporter gene assays indicated that L-PGC1α coactivated the transcriptional activity of
HNF4α at the PCK1 promoter, we used ChIP and pulldown assays as additional approaches to directly demonstrate a physical interaction of L-PGC-1α with HNF4α. We transiently transfected HEK293 cells with expression plasmids encoding the Halo-tagged versions of PGC-1α, L-PGC-1α, and HNF4α and studied the recruitment of the respected fusion proteins to the PCK1 promoter. Using DNA fragments captured by HaloTag resins, we performed PCR with a primer pair amplifying a 177-nucleotide fragment spanning the predicted HNF4α binding site on the PCK1 promoter (39). Signals of the expected sizes were obtained for all three constructs, whereas no signal was noted in cells expressing solely the HaloTag protein (Fig. 8A). Next, we performed pulldown experiments with nuclear extracts from HEK293 cells transiently transfected with empty vector and Halo-tagged L-PGC-1α or HNF4α expression plasmids. HNF4α clearly was present in protein complexes isolated from cells expressing Halo-tagged L-PGC-1α but not from cells transfected with the empty vector or the Halo-tagged HNF4α expression plasmid (Fig. 8B). The latter result was expected as the Halo-tagged protein covalently binds the HaloTag resin.

Previous studies have shown that coactivation of PPARα and glucocorticoid receptor by PGC-1α requires an intact AF-2 domain (10, 47, 48). To determine whether coactivation of HNF4α by L-PGC-1α also is dependent on AF-2 function, we mutagenized this domain in HNF4α and its Halo-tagged version (49) and studied its effect on PCK1 promoter activation. In

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

**FIGURE 6.** Induction of L-PGC-1α mRNA by dexamethasone, 8-Br-cAMP, and various transcription factors in HepG2 cells. A, HepG2 cells were incubated with 1 μM dexamethasone or 100 μM 8-Br-cAMP for 6 h, and mRNA levels were measured by quantitative real-time RT-PCR. B, correlation of hepatic L-PGC-1α and PCK1 transcript levels in humans. C, L-PGC-1α promoter-reporter constructs of various lengths were transfected into HepG2 cells in equimolar amounts and incubated as in A prior to measurement of reporter gene activities. D, mutagenesis (Mut) of glucocorticoid response element (GRE) or the CREB binding site (CREBP) in the 0.542-kb promoter construct abrogates its activation by glucocorticoids or 8-Br-cAMP in HepG2 cells. Columns (error bars) are means (S.D.) of quadruplicate determinations; asterisks within bars denote p < 0.01 versus control. E, transactivation of L-PGC-1α and PGC-1α promoter-reporter constructs by various transcription factors with reported activity in liver. Asterisks within bars denote p < 0.01 versus control. AU, arbitrary units; Prom-LUC, promoter-luciferase.
Liver-specific Human PGC-1α Isoform

Alternative pre-mRNA processing and/or transcript initiation substantially enhances the complexity of mammalian transcriptomes as multiple transcripts and proteins with distinct functions may be produced from a single gene locus. A system-
atic 5′-end analysis of the human transcriptome using the cap analysis of gene expression approach showed that 58% of human protein-coding transcriptional units had one or more alternative promoters (50). We show here that an alternative promoter of PGC-1α is used in human liver to produce a novel transcript that encodes a biologically active protein, termed L-PGC-1α.

The structure of the novel transcript was deduced from data obtained by several complementary methods. Transcriptional start sites identified by RLM-RACE were consistent with predictive promoter algorithms, results from Northern blots, and the demonstration of promoter activity in the immediate upstream region. Wild-type and liver-specific transcripts can be initiated from two adjacent sites in TATA-less promoters. The novel exon 1L is spliced to the regular acceptor site of exon 3 and contains wild-type exons 3–13 in a regular order.

Apart from liver, exon 1L is transcribed in testis albeit at a lower level. However, exon 1L-containing transcripts in testis may differ from the respective liver transcripts as NCBI dbEST entry BX105309 indicates another exon upstream of exon 1L. Testis and brain express the largest amount of variant transcripts (51). Although exon 1L is not expressed in human brain, we identified two major variant brain transcripts initiated from promoters distinct from the liver-specific promoter. Thus, tissue-specific differences in core promoter recognition factors (52) may play a role in PGC-1α transcription. L-PGC-1α transcripts can undergo alternative 3′-splicing. The resulting transcript predicts a protein resembling NT-PGC-1α but devoid of the N-terminal activation domain. Whether such a protein that also lacks the mediator binding site is produced by the human liver or whether the respective transcripts have other functions or are targeted to nonsense-mediated mRNA decay (53) remains to be determined.

A taxonomic comparison indicated that all main interacting domains of PGC-1α are highly conserved across recently diverged mammalian species (16). Although exon 1L is located in an evolutionarily conserved region, L-PGC-1α transcript homologues were not detected in livers of several mammals. Thus, L-PGC-1α most likely reflects an adaption to more complex pathways in humans.

Differentially regulated transcription start sites frequently generate alternative N termini (54). L-PGC-1α was predicted to lack 127 amino acids at the N terminus. A protein of the expected size was translated in vitro and detected with specific antibodies in liver and HepG2 cells. The altered structure of L-PGC-1α likely results in functional changes defined by retained and deleted domains (Fig. 3F). The activation domain facilitating recruitment of SRC-1 and CREB-binding protein (55) has been mapped to the deleted region. Furthermore, the first of three LXXLL motifs, the nuclear export signals, and the GCN5 interaction site mapped to amino acids 1–97 of PGC-1α (56) are deleted in L-PGC-1α. Thus, wild-type and L-PGC-1α may differ in properties related to recruitment of chromatin-modifying factors, intracellular trafficking, coactivation of transcription factors, and possibly degradation and post-transla-

4 S. M. Soyal, T. K. Felder, S. Auer, P. Hahne, H. Oberkofler, and W. Patsch, unpublished observations.
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A

ChIP: Halo

B

IP: Halo

FIGURE 8. L-PGC-1α is recruited to PCK1 promoter and physically interacts with HNF4α. A, chromatin from HEK293 cells transfected with Halo-tagged expression plasmids as indicated was captured using HaloTag resin and analyzed by PCR amplifying a fragment spanning the predicted HNF4α binding site in the human PCK1 promoter. B, nuclear extracts from HEK293 cells transfected with Halo-tagged expression plasmids as indicated were subjected to pulldown with HaloTag followed by immunoblotting with a monoclonal HNF4α antibody. Histone-3 (H3) antibody was used as a loading control for nuclear extracts. WB, Western blot; neg., negative; IP, immunoprecipitation.

Protein modifications. PGC-1α shares an N-terminal activation domain, an arginine-serine-rich domain, and an RNA binding domain with the other protein family members PGC-1β and PGC-related coactivator (17). The homology of L-PGC-1α with both of these other proteins is therefore restricted to the C terminus.

L-PGC-1α is located mainly in the nucleus and therefore likely to be transcriptionally active. To this end, we used human systems to delineate functional differences between PGC-1α and L-PGC-1α. The finding that L-PGC-1α coactivated PPARγ-mediated transcription was not unexpected because the docking surface for PPARγ is retained in the hepatic isoform. Furthermore, both proteins coactivated PPARα- and HNF4α-mediated transcriptional activation, but only PGC-1α effectively coactivated LXRα at the SREBP-1c gene promoter. As the interaction of PGC-1α with HNF4α is central to hepatic gluconeogenesis, we extended the studies of coactivation of HNF4α by L-PGC-1α and used ChIP and pulldown assays to demonstrate a direct physical interaction between the two proteins at the PCK1 promoter. In addition, mutational studies suggested that coactivation of HNF4α by L-PGC-1α requires an intact AF-2 site. The crystal structure of HNF4α in a complex with a PGC-1α fragment containing all three LXXLL motifs, also termed NR boxes, has been resolved recently (57). Only one of the three LXXLL motifs was bound at the canonical binding pocket. However, the bound LXXLL motif was not a selected box but represented an averaged structure of more than one NR box. Functional studies showed a main role of NR boxes 2 and 3 in binding and coactivation of HNF4α. As NR boxes 2 and 3 are retained in L-PGC-1α, its coactivation of HNF4α is plausible. An intact NR box 2 also was necessary for coactivation of LXRα, but LXRα and other NR box 2-dependent NRs such as PPARα and glucocorticoid receptor coactivated by PGC-1α differed in that coactivation of the former was not affected by removal of a repressor binding to NR box 3 (30, 31, 58, 59). Small differences in NR binding pockets can create local environments that allow NR-specific recruitment of coactivators (57). Therefore, it is possible that NR box 1 and/or additional factors recruited via the N-terminal region (deleted in L-PGC-1α) are required for effective coactivation of LXRα.

PGC-1α plays a central role in the metabolic adaptions of the liver to fasting. In PGC-1α-deficient mice, the program of hormone-stimulated gluconeogenesis is defective, whereas constitutively activated gluconeogenesis is maintained (60). Our studies in HepG2 cells suggest that the hormonal changes that increase hepatic PGC-1α expression also enhance L-PGC-1α expression. Like PGC-1α mRNA, L-PGC-1α mRNA increased in response to glucocorticoids and CREB signaling. As suggested by the promoter studies, FOXO1 may play an even greater role in L-PGC-1α than in PGC-1α transcription. Furthermore, the coactivation of PCK1 transcription and the strong correlation with PCK1 transcripts also argue for a role of
Like PGC-1α, L-PGC-1α coactivated PPARα-mediated transcription. As PPARα trans-activated the L-PGC-1α promoter, a feed forward loop may be created that enhances fatty acid oxidation, thereby supporting hepatic ATP production in the fasting state.

In conclusion, we have identified an alternative PGC-1α transcript that appears to be specific for human liver and encodes a functional protein that lacks 127 amino acids at the N terminus. In vivo correlations between L-PGC-1α transcript levels and hepatic mRNA levels encoding distinct transcription factors together with our trans-activation studies in HepG2 cells suggest overlapping transcriptional networks regulating the expression of L-PGC-1α, PGC-1α, and their downstream targets. Collectively, our data suggest a role of L-PGC-1α in hepatic gluconeogenesis, but further studies are needed to rationalize differences in regulation and function between PGC-1α and L-PGC-1α.

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