C/EBPβ Reprograms White 3T3-L1 Preadipocytes to a Brown Adipocyte Pattern of Gene Expression

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C/EBPβ is a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors that play important roles in tissue-specific gene regulation. C/EBPβ is expressed in a number of tissues including brown adipose tissue, muscle, liver and brain. It may play a role in the stimulation of oxidative capacity via its interaction with the NRF1 and -2 family, which regulates mitochondrial biogenesis and the expression of mitochondrial oxidative enzymes (12). The nuclear receptor coactivator, PPARγ coactivator-1α (PGC-1α) has been shown to be involved in the expression of the BAT phenotype (11). PGC-1α is expressed in a number of tissues including brown adipose tissue, muscle, liver and brain (11). It may play a role in the stimulation of oxidative capacity via its interaction with the NRF1 and -2 family, which regulates mitochondrial biogenesis and the expression of mitochondrial oxidative enzymes (12). PGC-1α is induced by exposure to cold or adrenergic stimulation in BAT but not WAT, and ectopic expression of PGC-1α induces expression of UCP1 in WAT cells (11). PGC-1α is also expressed in non-adipose cells under circumstances in which increased metabolic energy expenditure is favored, for example in exercise-conditioned muscle tissue (13) or in the liver in response to glucagon stimulation during fasting (14).

The expression of PGC-1α is cAMP-dependent in liver and BAT, and a major role of PGC-1α is to augment cAMP-mediated transactivation of effector genes such as UCP1 in BAT, or PEPCK in the liver (11, 13). cAMP induction of PGC-1α appears to depend primarily on a single conserved cAMP response element (CRE) found within 200 bp of the transcription start site (14, 15). Although this proximal CRE can drive
PGC-1α expression in both BAT and liver, additional unidenti-
tified mechanisms operate to silence cAMP-inducible PGC-1α
expression in WAT, even though a range of other WAT
expressed genes are cAMP-regulated (16).

A genome-wide analysis of CREs in the human genome has
revealed that CREB-binding protein (CBP) and phosphorylated
CREB (phospho-CREB) are widely bound to CREs without
transactivation (17). Despite the association of phospho-CREB
with a number of coactivators (CBP/p300), gene activation is
weak and appears to require additional phospho-CREB regula-
tory partners for stable recruitment of the preinitiation com-
plex. Studies on the PEPCK and interleukin-10 promoters have
demonstrated an interaction between CRE and C/EBP binding
sites in conferring tissue-specific and differentiation-depend-
ent responses to cAMP (18–20).

To investigate the differential activation of PGC-1α and
UCP1 gene expression by cAMP in WAT and BAT, we studied
the control of the PGC-1α proximal promoter in HIB1B and
3T3-L1 adipogenic cell lines. Here we report that the CRE-
containing proximal region of the PGC-1α promoter is suffi-
cient to confer adipose cell-specific cAMP inducibility. We fur-
ther report for the first time C/EBPβ expression and binding to
the CRE as the mechanism that is able confer a BAT gene
expression pattern on 3T3-L1 white preadipocytes.

EXPERIMENTAL PROCEDURES

**Plasmids**—Firefly luciferase reporter gene constructs con-
taining 1873 bp (1873PGC1α-pGL3), 890 bp (890PGC1α-
pGL3), and 264 bp (264PGC1α-pGL3) from the region upstream of the rat PGC-1α transcription start site were gen-
erated using the pGL3-Basic vector (Promega). These frag-
ments were generated by PCR using primers 1873PGC1α sense
(GTACCGGTACATTCTGCTTTGAAG), 890PGC1α sense
(GTACCGGTCTGGAGCCTATGAGAGCC), and
264PGC1α sense (GTACCGGTATGGTCTTTGCCTTCTCTA-
TAT) and antisense (GTCTCGAGCACCACCTCAATC-
CACCCTGAC) that had MluI or XhoI restriction sites at the
5’-end and were digested with the restriction enzymes XhoI and
MluI restriction sites at the
5’- and were digested with the restriction enzymes MluI and
XhoI to generate appropriate protruding ends. The pGL3-
Basic vector (Promega) was also digested with the same
enzymes, and the inserts were then ligated into these vectors.

Site-directed mutagenesis on the luciferase promoter (264PGC1α-pGL3) was performed using the QuickChange site-
directed mutagenesis kit (Stratagene). To mutate the CRE the
original sequence TGACGTCA was mutated to GACTACTG.
Successful mutagenesis was monitored by sequence analysis
(John Innes Sequencing Centre, Norwich, UK).

The pMSVC/EBPa (rat), pMSVC/EBPβ, and pMSVC/EBPβS
(mouse) expression plasmids, which contain the respective
cDNAs under the control of the mouse sarcoma virus (MSV)
long terminal repeat, were kindly provided by S. McKnight
(University of Texas Southwestern Medical Center, Dallas).
The mock plasmid pcDNA3 was from Invitrogen. The CRE
positive vector (6CRE-pGL3) was a kind gift from Robert
Newton (Dept. of Thoracic Medicine, National Heart and Lung
Institute, Imperial College School of Medicine, London).

**Cell Culture, Transfection, and Luciferase Assay**—3T3-L1
cells (ECACC) and HIB1B cells (kindly provided by B.
Spiegelman) were maintained in Dulbecco’s modified Eagle’s
medium with 10% fetal bovine serum (Invitrogen) in 5% CO2.
For differentiation, cells were cultured to confluence (day 0)
and then exposed to the differentiation mixture (0.5 mM
3-isobutyl-1-methylxanthine, 250 nM dexamethasone, 20 nM
insulin, 1 mM T3). After 48 h, cells were maintained in medium
containing 20 nM insulin and 1 mM T3 until day 7 for harvest.

The reporter plasmids were co-transfected using 3 μl of
FuGENE 6 (Roche Applied Science)/μg of DNA or 2 μl of Lipo-
fectamine 2000 (Invitrogen)/μg of DNA into 3T3-L1 and
HIB1B cells at 80% confluence in combination with pMSV/EBPa,
pMSVC/EBPβ, pMSVC/EBPβS, or pcDNA3 as a control.
The pRL-SV40 (from Promega) that carries Renilla luciferase
was also co-transfected as an internal control for monitoring
the transfection efficiency. At confluence (approx 24 h later),
cells were treated with forskolin under serum-free conditions,
and after 12 h cells were harvested and luciferase activities
analyzed using the Dual-Luciferase assay kit (Promega) as recom-
manded by the manufacturer. Values were expressed relative to
the control Renilla to allow for differences in transfectional
efficiency; there was no difference between cell type and treat-
ments on the average Renilla values.

**Electrophoretic Mobility Shift Assay and Supershift Assay**—
Preparation of nuclear extracts for electrophoretic mobility
shift assays was performed in the presence of protease and pro-
tein phosphatase inhibitors using the nuclear extract kit from
Active Motif. 10 μg of nuclear proteins extracted from confluen-
t HIB1B or 3T3-L1 cells were incubated with ~106 cpm of
radiolabeled oligonucleotide in a 20-μl reaction for 30 min at
room temperature. The oligonucleotide spanned the PGC-1α-
CRE regulatory element: 5’-TGACGCGTACCATTCTGCTGTCTTGAAG-
GAGTTTGTGCA-3’ (CRE motif is in italics). Specific binding
was established by co-incubating with 100-fold excess of either
unlabeled oligo or oligo containing a mutated (underlined) CRE
motif 5’-TGACGCGTACCATTCTGCTGTCTTGAAG-
GAGTTTGTGCA-3’. Nuclear proteins were incubated with antibodies that indicated for 20 min prior to incubation with the radiolabeled
oligonucleotides. Antibodies against CREB and C/EBPβ (LAP)
were purchased from New England Biolabs, C/EBPa from
Active Motif, and C/EBPβ from Santa Cruz Biotechnology.
Competing unlabeled oligonucleotides were added 15 min
prior to the addition of labeled probe.

**Chromatin Immunoprecipitation (ChIP) Assays**—ChIP
assays were performed according to the manufacturer’s proto-
col (Upstate). Briefly, HIB-1B and 3T3-L1 preadipocytes were
maintained in Dulbecco’s modified Eagle’s medium with 10%
fetal bovine serum (Invitrogen) in 5% CO2 until confluence
(approx 48 h after plating). Following confluence, the cells were
then stimulated with vehicle solution (Me2SO) or forskolin (10
μM) for 1 h at 37 °C. The cells were then washed with ice-cold phos-
phate-buffered saline twice, harvested with scrapers, and lysed
with SDS lysis buffer. The whole-cell lysates were sonicated
with a Soniprep 150 for 30 s at the maximum setting. This was
repeated eight times with 1-min intervals between each 30-s
pulse, yielding chromatin fragments between 200 and 500 bp
in size. Lysates were centrifuged at 13,000 rpm (Eppendorf micro-

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FIGURE 1. Differential cAMP-inducibility of PGC-1α and UCP1 in HIB1B and 3T3-L1 cells. A, adipogenic gene mRNA at 0 (confluence) and 7 days after induction to differentiate. B, UCP1 mRNA at 0 and 7 days after induction to differentiate and treated with or without forskolin for 3 h. C, PGC-1α mRNA at 0 and 7 days after induction to differentiate and treated with or without forskolin for 3 h. Values were analyzed by quantitative real-time PCR and normalized against β-actin expression. Error bars represent S.E. of triplicate observations of one of three independent experiments.

Western Blotting—Cells were washed twice with ice-cold phosphate-buffered saline, lysed in modified radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 37 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5% SDS, and 0.5% sodium deoxycholate), and harvested with scrapers. The whole cell lysates were centrifuged at top speed for 15 min after 30 min of incubation on ice, and supernatants were mixed with 2× Laemmli sample buffer. An aliquot of 40–50 μg/well was fractionated by SDS-PAGE on 10% acrylamide gels and blotted onto polyvinylidene difluoride membrane. After 1 h of blocking in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) nonfat milk powder, the membrane was incubated in fresh buffer with the appropriate antibody for 1 h at room temperature. The antibodies used were: 1:1000 dilution of anti-C/EBPα, 1:500 dilution of anti-C/EBPβ and anti-C/EBPδ, and 1:250 dilution of anti-actin (Sigma). The antigen-antibody complex was detected by incubating the membrane for 1 h at room temperature in a buffer containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Cell Signaling) and visualized with SuperSignal West Pico blotting substrate (Pierce, Perbio Science, Cramlington, UK).

Real-time PCR—Total RNA was isolated from cells by TRI reagent (Sigma). Quantitative RT-PCR was performed using SYBR Green (Qiagen) according to the manufacturer’s instructions in Rotor Gene 3000 (Corbett Research). The sequences of the primers used for real-time PCR were: PGC-1α sense (GCCGCGTGTGATTTAGGT) and antisense (AAAACCTTCAAAGCGGTCTCTCAA), UCP1 sense (CCTGGCTCTCTCGGAAACAAA) and antisense (TGGAGCAGGTGGCGCTATC), PGC-1β sense (ACGACGAGAGCGCCGG) and antisense (GGAAGCCGGCGACAAG) and antisense (GGCTCGGGCACGTCTT), C/EBPα sense (CAGCACGAGGGCCTTTCT) and antisense (TCGCCGGGCCGCCCAGTC), adipsin sense (GAGACGAGAGGCAGG) and antisense (GAGACGAGAGCGCCGTCGATG), aP2 sense (AACACCGGATCTTCTG) and antisense (ATCCACGCACTTCCAG). Thermal cycler conditions were initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. The melting temperatures of the products were 82°C, 83°C, and 82°C for PGC-1α, UCP1, and aP2, respectively.
sequences. The TESS analysis (www.cbil.upenn.edu/tess) was used to predict transcription factor binding sites. All of the positions in the promoter refer to the translation start site of the rat PGC-1α gene.

RESULTS

Differential cAMP Induction of PGC-1α and UCP1 Expression in Confluent and Differentiated HIB1B and 3T3-L1 Cells—HIB1B and 3T3-L1 cells were induced to differentiate into adipocytes by treating confluent cells with a standardized hormonal mixture as described under “Experimental Procedures.” Differentiation status was verified by oil-red “O” staining (data not shown) and adipogenic gene expression profiling by quantitative RT-PCR (SREBP-1c, aP-2, adipins, UCP1, PGC-1α; Fig. 1). To stimulate cAMP accumulation, forskolin (10 μM) was added to confluent cells immediately prior to (0 days) or 7 days following exposure to the differentiation mixture. Preliminary studies were performed with HIB1B and 3T3-L1 cells to establish the optimal time (3 h) for incubation with forskolin. In HIB1B cells, forskolin treatment increased UCP1 expression by ∼50-fold in undifferentiated cells and by 1000-fold after 7 days of differentiation (Fig. 1B). By contrast, a lower base-line level of UCP1 expression was observed in 3T3-L1 cells, and forskolin had no effect on expression, regardless of differentiation status (Fig. 1B). Qualitatively similar differences in PGC-1α expression were also observed; higher basal expression levels in HIB1B cells, coupled with a pronounced inductive response to forskolin in both undifferentiated and differentiated states but no responses to forskolin were observed in 3T3-L1 cells at 0 and 7 days of differentiation (Fig. 1C). Responses to cAMP stimulation using 3-isobutyl-1-methylxanthine were similar to those observed for forskolin (results not shown). These results demonstrate that cAMP induction of PGC-1α and UCP1 mRNA can be observed in undifferentiated confluent HIB1B but not 3T3-L1 cells.

Cell-specific Expression of PGC-1α Promoter-Reporter Constructs: The Proximal 264 bp Is Sufficient for Specificity—To analyze the transcriptional mechanisms responsible for these cell-specific differences in PGC-1α expression, different lengths (from 264 to 1873 bp upstream of the predicted transcription start site) of the rat PGC-1α promoter were cloned into the luciferase reporter vector pGL3. These constructs were used in transient transfection experiments in HIB1B and 3T3-L1 cells. Preliminary experiments established that promoter activity measurements were optimal at 24 h after adding the hormonal mixture (differentiation mix) that is used to differentiate HIB1B and 3T3-L1 cells; these measurements were compared with levels in control undifferentiated Me2SO-treated cells (Fig. 2). In HIB1B cells, the addition of the differentiation mix increased luciferase expression from constructs containing all three lengths of the PGC-1α promoter by 4–5-fold (Fig. 2A). By contrast, in 3T3-L1 cells the differentiation mix did not elicit any increase in expression for any of the promoter constructs (Fig. 2B).

This result suggested that factors within the proximal promoter region, encompassed by the 264-bp construct, accounted for cell-specific differences in PGC-1α induction by cAMP. This short proximal fragment exhibits very high sequence conservation across species (98% in rat/mouse/human) and contains a CRE, previously highlighted by studies of PGC-1α expression in hepatic cells (14). We therefore performed experiments in undifferentiated cells under serum-free conditions to analyze the effects of forskolin on the expression of the 264-bp promoter-reporter construct (Fig. 3, A and B). Under these conditions, forskolin induced reporter expression in both cell lines, but as predicted from the first experiment, the effect was markedly greater in HIB1B cells than in 3T3-L1 cells.

These results led us to consider the relative involvements of the core CRE and adjacent flanking regions in the cAMP-inducibility of the 264-bp construct. Site-directed mutagenesis of the CRE at positions −183 to −175 abolished completely the effect of forskolin on the proximal PGC-1α promoter-reporter activity in both cell types (Fig. 3, A and B). These data indicate that the CRE on the proximal PGC-1α promoter is able to give widely differing response characteristics to a cAMP-mediated stimulus in the two different adipocyte cell contexts. Importantly, this result was not due to the different sensitivities to forskolin, or the PKA pathway in the two cell types, because a luciferase reporter construct driven by six CREs in tandem
A CRE on the 264-bp PGC-1α promoter is responsible for the adipose cell-specific response to a cAMP stimulus. A, 264PGC1α-pGL3 or the same construct containing a mutated CRE assayed after transient transfection in confluent HIB1B cells and treated with or without forskolin for 12 h. B, PGC-1α promoter-reporter or the same construct containing a mutated CRE, assayed in transfected 3T3-L1 cells. C, luciferase construct (6CRE-pGL3) driven by a 6-CRE transfected in HIB1B and 3T3-L1 cells. Error bars represent S.E. of triplicate observations of one of three independent experiments.

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(21) was induced to the same extent in 3T3-L1 and HIB1B cells (Fig. 3C).

Previous studies have demonstrated that both CREB and ATF-2 can bind to the proximal CRE lying within the 264-bp region (14, 15). The C/EBP family of transcription factors has also been reported to modulate the activity of CREs in gene promoters (20). Therefore we performed gel shift experiments to identify the presence of CRE-binding proteins in nuclear extracts from HIB1B and 3T3-L1 cells. Specific binding of nuclear proteins to the PGC-1α-CRE oligonucleotide was demonstrated by either the disappearance of four bands when incubations included 100-fold excess of unlabeled oligonucleotide spanning the PGC-1α-CRE and the same oligonucleotide with the CRE mutated, respectively. Numbered arrows mark specific binding. B, chromatin immunoprecipitation demonstrates forskolin-dependent CREB and C/EBPβ binding to the CRE region of the PGC-1α proximal promoter in lysates from HIB1B cells. In 3T3-L1 cells only forskolin-dependent C/EBPβ binding is observed. Assays are of one of three independent experiments.

These data, along with those showing the cAMP induction of the CRE-positive control reporter in 3T3-L1 cells, suggested that the differential response in forskolin-induced PGC-1α expression between the two cell types was not related to the upstream sensitivity of the PKA pathway but rather to combinatorial transcription factor effects on the proximal CRE, which were able to confer adipocyte-specific differential expression of this promoter to cAMP.
The Role of C/EBP Isoforms in Cell-specific cAMP Inducibility of PGC-1α—Evidence of the involvement of the C/EBP family of transcription factors in the modulation of the CRE in the promoter of other genes (20), coupled with the crucial role played by C/EBPs in commitment of progenitor cells to the adipocytes lineage (16), led us to consider whether differential expression of C/EBP isomers might be involved in the cell context-specific CAMP inducibility of the 264PGC1α-pGL3 luciferase reporter construct.

To explore this possibility, we first used quantitative RT-PCR to compare expression patterns for three C/EBP isoforms (α, β, δ) in the two cell types during differentiation (Fig. 5A) and forskolin challenge (Fig. 5B). This showed that although there was no clear distinction between HIB1B and 3T3-L1 cells in the pattern of C/EBPα expression in response to either differentiation or forskolin, both C/EBPβ and C/EBPδ showed cell-specific differences. In the case of C/EBPβ, expression rose markedly during differentiation in HIB1B cells but did not change in 3T3-L1 cells. Additionally, both C/EBPβ and C/EBPδ were strongly induced by cAMP stimulation in confluent HIB1B compared with 3T3-L1 cells. These observations suggested that increased levels of C/EBPβ or C/EBPδ protein expression in HIB1B cells might be necessary for the induction of PGC-1α and UCP1 expression in response to cAMP stimulation during the BAT differentiation program.

To test the role of C/EBP transcription factors on PGC-1α gene regulation, we transfected both cell types with the 264-bp PGC-1α promoter-reporter construct and overexpressed the C/EBPα, β, and δ isoforms. We first confirmed successful overexpression of the C/EBP isomers by Western blot (Fig. 6A). C/EBPβ overexpression more than doubled the activation of the 264-bp PGC-1α promoter-reporter construct. Importantly, this effect was observed in both HIB1B and 3T3-L1 cells (Fig. 6B). Overexpression of C/EBPα and C/EBPδ had a less marked effect on PGC-1α promoter activity than C/EBPβ (Fig. 6B). Overexpression of the three C/EBP isomers failed to influence the forskolin-induced expression of the 6CRE-pGL3 vector (Fig. 6C). These data suggested that C/EBPβ was responsible for the activation of the proximal PGC-1α promoter without affecting the sensitivity of the upstream PKA pathway.

To test the hypothesis that the CRE site mediates C/EBPβ modulation of PGC-1α cAMP-inducibility, we compared the reporter activity of the wild-type –264 bp promoter construct with that of a similar construct in which the CRE was mutated. As shown previously, C/EBPβ overexpression stimulated both basal and forskolin-stimulated activity of the wild-type PGC-1α reporter construct in both HIB1B and 3T3-L1 cells (Fig. 7). Mutation of the CRE effectively abolished any potentiating effects of C/EBPβ overexpression in either HIB-1B (Fig. 7A) or 3T3-L1 cells (Fig. 7B). These data demonstrate that C/EBPβ overexpression rescues CAMP-inducible PGC-1α expression in 3T3-L1 cells through effects within the proximal CRE containing region of the PGC-1α promoter.

To verify whether the observations of C/EBPβ overexpression on the PGC-1α promoter also corresponded to changes of the endogenous PGC-1α mRNA expression and cell commitment, we transiently overexpressed C/EBPβ protein in the two cell types and assessed the impact on cAMP-induced PGC-1α and UCP1 gene expression. As predicted from our earlier experiments, forskolin increased PGC-1α and UCP1 mRNA expression in HIB1B cells expressing a control “empty” p-cDNA3 vector but not in 3T3-L1 cells (Fig. 8). Furthermore, overexpression of C/EBPα, C/EBPβ, or C/EBPδ in HIB1B cells elicited no further increase in PGC-1α or UCP1 expression in response to forskolin (Fig. 8).
FIGURE 6. Overexpression of C/EBPβ rescues PGC-1α proximal promoter transcriptional activity responses to cAMP in 3T3-L1 cells. HIB1B or 3T3-L1 cells were transiently co-transfected with an empty control vector p-cDNA3 or 264PGC1α-pGL3, p-MSV-C/EBPα, p-MSV-C/EBPβ, p-MSV-C/EBPδ, and 6CRE-pGL3 as indicated, and at confluence were treated with forskolin for 12 h. A, Western blot showing overexpression of C/EBP isomers. B, 264PGC1α-pGL3 promoter-reporter activity. C, 6CRE-pGL3 luciferase constructs driven by a 6-CRE in tandem. Error bars represent S.E. of triplicate observations of one of three independent experiments.
Remarkably, overexpression of C/EBPβ caused 3T3-L1 cells to exhibit a BAT pattern of gene expression, i.e. highly forskolin-inducible PGC-1α and UCP1 mRNA expression. This large response was specific to C/EBPβ, because overexpression of C/EBPα and C/EBPδ had either no effect or much less effect on the PGC-1α or UCP1 mRNA in either cell type (Fig. 8).

In conclusion, we have provided evidence that C/EBPβ plays a pivotal role in the adipocyte-specific cAMP-induced expression of PGC-1α and UCP1 and is able to reprogram white preadipocyte 3T3-L1 cells to a BAT pattern of gene expression.

**DISCUSSION**

PGC-1α expression in brown fat is increased during cold exposure due to adrenergic activation of the PKA pathway (11). Our initial studies demonstrated that the forskolin-induced increase in PGC-1α and UCP1 mRNA in differentiated HIB1B cells was also observed in confluent HIB1B cells, suggesting that these genes become inducible early in the brown adipogenesis program. As expected, forskolin did not stimulate either PGC-1α or UCP1 mRNA in differentiated or confluent 3T3-L1 white preadipocyte cells. Here we demonstrate that the differential response in cAMP-induced PGC-1α expression between HIB1B and 3T3-L1 cells is caused by interaction of C/EBPβ with the CRE on the proximal promoter of PGC-1α.

In gel shift experiments we found that nuclear extracts from both HIB1B and 3T3-L1 cells bound CREB and C/EBPβ, but not C/EBPα or C/EBPδ, to the CRE in the PGC-1α proximal promoter. ChIP assays confirmed that in HIB1B cells, C/EBPβ and CREB bound to the CRE region of the PGC-1α promoter in a forskolin-dependent manner. In 3T3-L1 cell lysates, C/EBPβ but not CREB binding was forskolin-dependent. These experiments suggested that C/EBPβ may play an important role in mediating the differential effects of cAMP stimulation on PGC-1α expression in HIB1B and 3T3-L1 cells.

Evidence to support this hypothesis came from the demonstration that overexpression of C/EBPβ increased forskolin-induced transcriptional activity of the 264-bp PGC1α reporter construct in both HIB1B and 3T3-L1 cells. Furthermore, overexpression of C/EBPβ rescued the suppression of forskolin-induced PGC-1α expression in 3T3-L1 cells assessed by both reporter assays and direct mRNA measurements. This effect was specific to C/EBPβ, because similar experiments with C/EBPα or C/EBPδ overexpression failed to elicit the same increased sensitivity to cAMP stimulation. Mutation of the CRE on the PGC1α proximal promoter abrogated adipocyte nuclear protein binding and the potentiating effects of C/EBPβ overexpression on transcriptional activity of the 264-bp PGC1α reporter construct in both the HIB1B or 3T3-L1 cells. Importantly, overexpression of C/EBPβ, but not C/EBPα or C/EBPδ, in 3T3-L1 cells enabled cAMP to induce UCP1 expression by 260-fold. These data argue that the programming of preadipocytes to a BAT lineage is due to C/EBPβ interacting with CREB at the CRE on the PGC-1α proximal promoter.

C/EBP-related proteins have been found to potentiate cAMP-inducible expression of the PEPCK promoter (19) in a tissue-specific and differentiation-dependent manner. In contrast with the present study, this required CREB and C/EBPα or C/EBPβ to bind to separate sites on the promoter. Leucine zipper transcription factors also interact as homo- and heterodimers with CREs. Regulation of the interleukin-10 (20) and pre-interleukin-1β (22) promoters and human immunodeficiency virus type 1 long terminal repeat (23) by cAMP involves heterodimer formation between CREB and C/EBPβ, suggesting that the specific profile of transcription factors is capable of inducing tissue-specific patterns of gene expression (14, 19, 24). Cooperation between CREB and C/EBPβ has been suggested in rat C6 glioma cells transfected with tandem CRE sites in a pCRE-Luc reporter construct (25), but we were unable to observe this in our adipogenic cell lines.

An alternative mechanism, consistent with our results, is that C/EBPβ binds to CREB inducing a conformational change that recruits additional CBP/p300 into the preinitiation complex. C/EBPβ-CREB binding at a site independent of the CRE leucine zipper and CBP binding domains has been demonstrated (25). C/EBPβ interaction with other proteins as well as...
subcellular localization, DNA binding, and transactivation potential are regulated by phosphorylation and require further investigation.

There is physiological evidence to support a role for C/EBPβ in BAT differentiation. Like PGC-1α, C/EBPβ expression in BAT is cold-inducible; during cold exposure, adrenergic stimulation and early development in rodents, C/EBPβ, but not C/EBPα or -δ, is increased in BAT but not WAT, in parallel with increases in UC1P and PGC-1α mRNA (26, 27). We have confirmed that PGC-1α, C/EBPβ, and UC1P mRNA increase in BAT but not WAT of cold-stressed mice (results not shown). Furthermore, in the present study we observed that C/EBPβ expression was increased in HIB1B but not 3T3-L1 adipocyte cell lines when challenged with forskolin to increase cAMP. C/EBPβ-/- mice have decreased brown adipose tissue and are cold-intolerant, but this is not because of a decrease in BAT UC1P expression (28). However, this is likely to be because of a compensatory increase in C/EBPδ expression (28), because double knock-out of C/EBPβ and -δ prevents BAT accumulation (10). In the present study, overexpression of C/EBPβ in 3T3-L1 cells increased forskolin-induced PGC-1α and UC1P expression although to a much lesser extent than with C/EBPβ overexpression (Fig. 8). Genetic ablation studies have demonstrated that C/EBPβ and -δ, but not C/EBPα, play critical roles in brown fat differentiation (10) but it is not clear whether C/EBPs play a direct role in the expression of UC1P.

An outstanding puzzle is how C/EBPβ can be responsible for transduction to a BAT phenotype when it has been demonstrated that a transient increase in C/EBPβ initiates the adipogenic differentiation program in 3T3-L1 white adipose precursor cells. We speculate that this may be explained by the temporal dynamics of C/EBPβ expression with sustained C/EBPβ exposure resulting in the brown phenotype. Transgenic mice expressing C/EBPβ under the control of the C/EBPα promoter have 60% more brown adipose tissue and reduced white fat cell size than their wild-type littermates (29). During white adipocyte differentiation, C/EBPα is continuously expressed after initiation by C/EBPβ, providing evidence that sustained C/EBPβ expression may be critical in committing cells to the brown phenotype (16). In summary, we have demonstrated that C/EBPβ plays a key role in regulating the expression of PGC-1α and UCP1 during early differentiation in adipocyte cell lines and in combination with adrenergic stimulation is able to reprogram white preadipocyte 3T3-L1 cells to a brown adipocyte lineage. These data provide new insights into the developmental mechanisms controlling cell differentiation and adipose tissue composition.

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