Sequestration of Thermogenic Transcription Factors in the Cytoplasm during Development of Brown Adipose Tissue*

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Transcription factors that regulate gene expression during adipogenesis also control the expression of genes of thermogenesis in brown adipose tissue, in particular, the mitochondrial uncoupling protein gene (Ucp1). There is evidence that a plasticity exists among adipocyte cultures that increases brown adipocyte character of white fat. To understand this process, we have characterized the changes in transcription that occur in interscapular brown adipocytes during development. We have found dramatic reductions in both DNA-binding activity to probes and immunoreactive protein for peroxisome proliferator-activated receptor, retinoid X receptor, CCAAT/enhancer binding protein, and cAMP-response element-binding protein regulatory motifs in nuclear extracts when mice reach adulthood. Exposure of adult mice to the cold, which reactivates Ucp1 expression, leads to a reaccumulation of factors in the nucleus. We propose that transcription factors are sequestered in the cytoplasm as mice age under conditions of reduced thermogenesis. Changes in isoform subtypes for peroxisome proliferator-activated receptor-γ and cAMP-response element-binding proteins indicate an additional level of control on gene expression during thermogenesis. The increased movement of the RIIβ protein kinase A regulatory subunit into the nucleus with age suggests a mechanism for regulating the phosphorylation of transcription factors in the nucleus in response to the thermogenic requirements of the animal. Nuclear factor-κB has been used as a model to demonstrate that the nuclear localization of transcription factors in brown fat are reduced during post-natal development. Furthermore, it was found by immunofluorescence that adrenergic stimulation of primary adipocyte cultures causes an increase of both the protein kinase A catalytic α-subunit and nuclear factor-κB into the nucleus.

Brown adipose tissue (BAT) develops precociously in many species to provide adaptive thermogenesis for the maintenance of body temperature when the ambient temperature is reduced (1, 2). This function is most critical at birth, but as the animal matures, other strategies for protection from the cold become available and the need for brown fat nonshivering thermogenesis is reduced. It has also been proposed that brown fat is an important source of diet-induced thermogenesis (3); however, recent studies with uncoupling protein-1 (UCP1)-deficient mice have challenged this view (4). Exactly what happens to the brown fat as the need for its function wanes is uncertain, particularly in circumstances when the need for nonshivering thermogenesis may return. Because the activation of nonshivering thermogenesis is primarily mediated by adrenergic signaling (5), it is probable that a reduction in this signaling process represses the expression of genes of thermogenesis, especially those controlling mitochondrial function, including Ucp1 expression, and ultimately results in loss of brown adipocyte character (6, 7). Whether the brown adipocytes die or assume the morphology of a white adipocyte is unknown. However, because the reactivation of Ucp1 expression in the interscapular brown fat of adult mice occurs within minutes of being exposed to the cold (8), the transcriptional machinery must be available to respond to this physiological need.

The transcriptional regulation of Ucp1 gene is controlled by regulatory elements that have been shown to be critical for both white fat and brown fat adipogenesis. This includes members of the peroxisome proliferator activated receptor (PPAR), CCAAT/enhancer binding protein (C/EBP) and cAMP response element binding protein (CREB) families of transcription factors (9, 10). Pivotal to this regulation is the peroxisome proliferator activated receptor (PPAR) site in the distal Ucp1 enhancer for the formation of a transcriptional complex that includes PPARγ2, retinoid X receptor (RXR), and the PPAR coactivator (PGC-1α) (11–13). In its role in the regulation of Ucp1 and mitochondrial biogenesis, PGC-1α has become a molecular feature that distinguishes the regulation of brown adipocyte differentiation from that of the white adipocyte (13). Equally important for the regulation of Ucp1 are half-site cAMP response elements (CRE) in both the proximal promoter and the distal enhancer that are sites of interaction with CREB; mutations to these sites abolish expression in transient expression assays (14–16). Recent work has underscored the critical importance of CREB in activation of C/EBPβ to initiate the adipogenic differentiation program through mitotic clonal expansion.2 The role of PPARγ in adipogenesis has been extensively documented for both tissue culture and in vivo models of adipogenesis and brown adipocyte expression (17, 18). In addition, interactions between PPARγ and members of the C/EBP family members are critical for adipogenesis (19), and although C/EBPα seems not to be required for brown fat expression (20), C/EBPβ and -δ expression are critical for brown

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fat differentiation (21). It is not clear whether the C/EBPs are directly involved in the regulation of Ucp1. Also located in the Ucp1 enhancer region is a site for regulation by the thyroid hormone receptor (22) which links Ucp1 regulation to the potential influence of thyroid hormone on thermogenesis (23).

The analysis of preadipocyte differentiation in cell culture has been a powerful tool for the identification of the components of signaling and transcription pathways of adipogenesis; however, there are features of adipocyte physiology that cannot be elucidated by in vitro studies. In particular, we are interested in mechanisms determining transformation of white to brown fat: a phenotype of adipose tissue that depends on anatomical location of the fat pad, developmental age, nutritional status and genetic background (8, 24, 25). UCP1 is first detected in interscapular brown fat of the 19-day mouse fetus, whereas the BAT differentiation, evident by increasing mitochondria, was first detected at 15 to 16 days of fetal development (26). In adult animals, chronic stimulation of β-adrenergic receptors by cold exposure or β-agonist treatment results in increased Ucp1 expression (27, 28) followed by hyperplasia of BAT as well as recruitment of brown adipocytes in white adipose tissue (25, 29–31). Accordingly, we asked the question what happens to the transcription machinery in the brown fat during development from the fetus to the adult and what occurs to this apparatus in adult brown fat during reactivation of thermogenesis when the animal is again challenged by the cold. We found a dramatic reduction in adipogenic transcription factor DNA-binding activity in nuclear extracts during development of interscapular brown adipose tissue. The mechanism underlying this loss of transcriptional DNA-binding activity was not because of reductions in either total mRNA or protein, but rather to major changes in the cytoplasmic and nuclear localization of the transcription factors. In contrast, the amount of the RIIβ-protein kinase A (PKA) regulatory subunit in nuclear extracts increases as the mice reach maturity. When adult mice are re-exposed to the cold, transcription factors return to the nucleus. In addition, cold activation causes changes in cellular distribution and expression of isoform subtypes for CREB and PPAR-γ. Accordingly, changes in the phosphorylation state of transcription factors together with nuclear localization are part of a mechanism involved in the regulation of thermogenesis.

**MATERIALS AND METHODS**

**Animals and Treatments—**C57Bl/6j (B6) mice were kept on a 12-h light/12-h dark cycle and provided ad libitum with food and water. Interscapular brown adipose tissue was isolated from fetal mice and adult male mice at indicated times after mating or birth, respectively. For cold exposure, two or three mice per pen were placed in the cold (4°C) for 1 or 7 days prior to isolation of BAT.

**Brown Adipocyte Primary Culture and Pulse-chase Experiment—**Primary cultures from the stromal vascular fraction of interscapular brown adipose tissue were differentiated with DIM medium (1 μM dexamethasone, 0.5 mM methylisobutylxanthine, and 1.7 μM insulin) for 3 days. Cells were deprived of methionine for 30 min, labeled with [35S]methionine (Amersham Biosciences) for 60 min, and after 3 washes with chase medium containing cold methionine, treated with or without 1 μM norepinephrine (Sigma) in Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum for the indicated periods of time. Cells were lysed in 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate, 1% SDS containing protease inhibitor of thermogenesis.

**Preparation of nuclear extracts—**Cells were then washed with 10% calf serum in PBS and incubated at room temperature for 1 h with gentle rocking. Cells were washed 3× with 10% calf serum in PBS and incubated at room temperature for 1 h after adding fluorescence-labeled secondary antibody (FITC-conjugated anti-rabbit IgG from Sigma, 1:1000). Cells were washed 3× with 10% calf serum in PBS and covered with mounting solution and a cover glass until examined by fluorescence microscopy.

**Reverse Transcription (RT)-PCR Cloning and Quantitation of CREB Isoforms—**cDNA from interscapular brown adipose tissue of 14-day-old mice was generated using SuperScript II Choice system according to the manufacturer’s protocol (Invitrogen); the cDNA was then subjected to PCR amplification using primer pairs 5’-ceggactAGAATG-GAAGCAGCGAACA (the initiation codon for CREB is shown in bold), TACATCGGCGTGGCGG (C/EBP), GCCATCCGGCAGCC (the stop codon for CREB is shown in bold), and the XhoI site in lowercase letters is underlined. PCR products were subcloned into pBlueScript II SK (+) (Stratagene, La Jolla, CA) using EcoRI and XhoI restriction enzyme sites and then verified by DNA sequencing using T7 and T3 primers. To evaluate expression of CREB isoforms in BAT after cold exposure, ISSCRIP stained with 2% norepinephrine (Santa Cruz Biotechnology, Santa Cruz, CA). After a 24-h incubation at 4°C under gentle agitation, 20 μl of protein A-agarose (Santa Cruz Biotechnology) were added to each labeled reaction and agitated for 30 min at 4°C. The beads were collected by centrifugation for 1 min at 3000 × g and were successively washed in 1 ml of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA. Proteins in the immunoprecipitate were separated by SDS-PAGE and analyzed by autoradiography.

**Protein Isolation and Western Blotting—**Preparation of nuclear extracts was performed as described previously (32). Proteins from nuclear extracts (10 μg) or the total tissue fraction (200 μg) were separated on a 15% polyacrylamide gel and then transferred onto nitrocellulose membranes (Millipore, Bedford, MA). The membranes were incubated with antibodies against CREB (Santa Cruz Biotechnology), phospho-CREB (Cell Signaling Technology), PPAR-γ, CREB/NFκB or β-actin (Abcam, Cambridge, MA) according to the manufacturer’s protocol. Antibodies were kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center). Bands were visualized using the enhanced chemiluminescence reagent after application of horseradish peroxidase-conjugated antibody (Amersham Biosciences) or Odyssey imaging system (LI-COR Bioscience, Lincoln, NE) with fluorescent-labeled secondary antibody (IRDye800TM or Cy3.5), according to the manufacturer’s protocol.

**Immunofluorescence Cell Staining—**HIB-1 cells and primary cultures from interscapular brown adipose tissue and inguinal white adipose tissue were seeded on Lab-Tek chamber slides (Nalgé Nunc International, Naperville, IL). Cells were treated with 1 μM norepinephrine for 30 and 60 min and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were washed 3× with 10% calf serum in PBS for 5 min each, and then primary antibody in 10% calf serum containing 0.2% saponin in PBS was applied and incubated at room temperature for 1 h with gentle rocking. Cells were washed 3× with 10% calf serum in PBS and incubated at room temperature for 1 h after adding fluorescence-labeled secondary antibody (FITC-conjugated anti-rabbit IgG from Sigma, 1:1000) and Fluor 488 anti-mouse IgG from Molecular Probes). Cells were washed with 10% calf serum in PBS and covered with mounting solution and a cover glass until examined by fluorescence microscopy.

**Electrophoretic Mobility Shift Assay (EMSA)—**Electrophoretic mobility shift assays were performed as described previously (32). To prepare probes for EMSA, sense and antisense oligonucleotides were synthesized (Qiagen, Alameda, CA) as follows: PPRE, GATCTTGTGACCTTGGTCTCTTTGTAAG; CREB, TGCAAGTGGGCACTGCTG; RXR, AGCCTGACGTCTAGGTGCAGACTC; CRE, TTGCTGTAGCTCAGAGGA; CRE2 (mouse UCP1), TGAACATTGCTGTACCTTTT. 5 μg of nuclear extract were incubated initially for 10 min at room temperature in 29 μl of 20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1.5 μg of poly(dA-dT), and 5 mM MgCl2. The ability of specific antibody to supershift or block the DNA-protein complex was used to evaluate the specificity of interactions of proteins in nuclear extracts with probes, 1 μl of antibody was added to the reaction mixture and incubated at room temperature for 30–45 min. The reaction was then incubated for an additional 20 min after adding 32P-labeled probe (4 × 105 cpm/μl) with or without an unlabeled competitor. The reaction was electrophoresed on a 6% polyacrylamide gel in 0.5x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3), then the gel was dried and exposed to a PhosphoImager screen (Molecular Dynamics, Piscataway, NJ).

**Quantitative Real-time PCR—**Total RNA was isolated from brown adipose tissue using TRIzol reagent according to the manufacturer’s instructions (Molecular Research Center, Inc., Cincinnati, OH). Real-time quantitative RT-PCR was performed as described previously (33).
Briefly, RNA was diluted to 20 ng/μl in formamide and then to a 1:100 dilution (0.2 ng/μl) in deionized water just before use. 10 μl of diluted RNA (2 ng) was used in each 50-μl reaction with single-reporter measurement using the ABI Prism 7700 and 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The sequences for the primers and fluorescence probe (5′/3′) are shown in Table I. A standard was prepared as described previously (33). RNA from liver was used as a standard for PPARα. Both standards and samples were run in duplicate, and each transcript level was adjusted to the cyclophilin level of the sample.

RESULTS

Adipogenic Transcription Factors during BAT Development—Functional activity of adipogenic transcription factors in brown adipose tissue during fetal and post-natal development of mice was measured with EMSA. Binding activity of nuclear proteins to consensus binding sites (see “Materials and Methods” for the sequences) for PPARs, RXR, and C/EBPα were maximal in the 19-day fetus and remained high until 1 month of age, when it decreased dramatically. Similar binding profiles were observed for CRE and the half-site CRE (CRE2) from the mouse Ucp1 gene promoter (Fig. 1A). The specificity of binding was confirmed by the reduced signal intensity after the addition of an excess amount of cold probe and antibody to supershift or blocking of the DNA-protein complex to nuclear extracts from BAT of the 19-day fetus (Fig. 1B). Although BAT can be detected macroscopically by the 16th day of fetal development (26), we could not isolate sufficient amounts of nuclear extracts until the 19th day of fetal age (data not shown). The changes in binding activity of transcription factors associated with Ucp1 expression suggest that either transcriptional or post-transcriptional/translational modifications of these factors occur during development. To explore further the molecular basis for the loss of binding activity with age, the levels of mRNA and protein for the transcription factors were determined in total cell lysates and in nuclear extracts and related to the changes in expression of Ucp1. Expression of Ucp1 increased markedly between the 19th day of fetal development and 8 days of age at both the mRNA (Fig. 2A) and protein levels (Fig. 2B, left). Thereafter, expression decreased (note the log scale on the y-axis after break in Fig. 2A), although not to levels below that of the 19-day fetus, as was observed for transcription factor binding activity by EMSA. This direct correlation between Ucp1 mRNA and its protein was not followed by the transcription factors. In contrast to the dramatic reduction in nuclear protein binding activity, levels of mRNA for all of the transcription factors show a gradual increase that began during fetal development and continued during post-natal development (Figs. 2A and 3A). No single transcription factor showed the dramatic reduction in expression at the mRNA level that was seen for DNA-binding activity (Fig. 1). Accordingly, there is no correlation between variation in the functional activity of transcription factors during development and their respective mRNA levels.

The levels of transcription factor protein in total cellular extracts and nuclear extracts were estimated by immunoblot analysis. These results (shown in Figs. 2B, 3B, and 4D) reveal major reductions in the amount of the transcription factors in the nucleus as the mice age, despite high levels of proteins in total cell extracts. In addition, a developmental switching of isoform types occurred for PPARγ and CREB. Similar amounts of PPARγ1 and γ2 were detected in total protein extracts from BAT of the 19-day fetus. Subsequently, PPARγ2 decreased (Fig. 2B) until PPARγ1 became the dominant isoform in BAT of adult mice. Levels of PPARα and PGC-1α protein in total cell extracts gradually increased after birth (Fig. 2B), generally in parallel with their respective mRNA levels. In general, the levels of protein in total cell extracts were consistent with the profile of their respective mRNA; however, this did not explain the huge loss in EMSA binding activity in nuclear extracts. Accordingly, protein levels of transcription factors were estimated in nuclear extracts by immunoblots. The virtual absence of UCP1, a cytoplasmic protein, in blots of nuclear extracts indicated that nuclear extracts were not contaminated with their respective mRNA; however, this known association with the nuclear pore complex also makes it a useful control for nuclear protein; however, its known association with the nuclear pore complex also makes it useful control for nuclear protein extracts (34, 35). Nuclear extracts from fetal and young mice contained much more of both PPARγ isoforms than adult mice, and more of PPARγ1 than PPARγ2. The pattern of expression for PPARα was different, because it showed a striking increase in protein levels during development and a fairly flat signal in the nuclear extracts. These results indicate that the amount of protein detected in nuclear extracts is directly correlated with changes in the binding activity of factors in nuclear extracts to their cognitive DNA-binding motifs. In addition, antibody supershift or inhibition of the DNA-protein complex demonstrated that PPARγ and C/EBPβ are the major transcription factors that bind to RXR/PPRE and C/EBP in brown adipose tissue, respectively. These binding patterns in EMSA are also consistent with their expression in nuclear extracts that showed expression levels of PPARγ and C/EBPβ higher than those of PPARα and C/EBPδ (Figs. 2B and 3B).

Expression of C/EBP during BAT Development—C/EBPα and C/EBPδ genes are transiently expressed in the early stage of adipose conversion in 3T3-L1 cells (36, 37). Mice lacking C/EBPβ and/or C/EBPδ showed impaired adipogenesis in both brown and white adipose tissues (21). In this experiment, we identified the expression pattern of C/EBPα and C/EBPδ during BAT development. Fig. 3A demonstrated that C/EBPβ mRNA increased after birth; however, no changes were detected in C/EBPδ mRNA (Fig. 3A). In contrast to changes in mRNA levels, C/EBPα and C/EBPδ protein in total cellular lysate and nuclear fraction were maximal in the 19-day fetus and gradually decreased after birth (Fig. 3B). Similar to the
PPARs, loss of nuclear binding activity for the C/EBPs seems to be directly related to the absence of the transcription factor in the nucleus.

Isoform-specific CREB Phosphorylation and Expression during BAT Development—Alternative splicing generates various CREB isoforms (38) that vary from tissue to tissue (39). To establish the distribution of CREB isoforms in brown adipose tissue, we cloned CREB cDNAs and determined the relative frequency of each isoform by DNA sequencing. PCR amplification of mouse cDNA from BAT with CREB gene-specific primers (see “Materials and Methods” for the sequences) showed two distinct bands of 1 kb and 0.8 kb, corresponding to CREBα/β/γ and CREBα isoforms, respectively (data not shown).

PCR products were subcloned into pBlueScript II SK(+) cloning vector (Stratagene), and the inserts from 57 independent clones were analyzed by sequencing. Consistent with the previous report (39), the major isoform in mouse BAT was CREBα (55.4%; see Fig. 4A). Surprisingly, 28.6% of CREB isoform in BAT was a thymus-specific CREB isoform (CREBβ/γ), which lacks the glutamine-rich domain (Q2/CAD) of CREBΔ (40). In addition, RT-PCR analysis with total RNA from various tissues of the mouse showed that the CREBβ isoform is highly expressed in the spleen and thymus, with relatively small amounts in BAT and muscle (data not shown).

To compare the binding affinity of CREB isoforms to CRE by EMSA, equal amounts of in vitro translated CREB isoforms were incubated with 32P-end-labeled CRE probe. As shown in Fig. 4B, in vitro translated CREB isoforms generated double-specific binding complexes in which the upper bands are homodimeric complexes of each CREB translation product, whereas the lower bands are heterodimeric complexes between complete and truncated CREB translation products. Therefore, we considered the upper bands as CREB-specific binding in the electrophoretic mobility shift assay. Fig. 4B demonstrated that the binding of CREB isoforms were not significantly different; however, in vitro de-phosphorylation analysis demonstrated that CREBΔ is most sensitive to calf intestinal phosphatase treatment, compared with the CREBα and CREBβ/γ isoforms (Fig. 1).
De-phosphorylation of CREBΔ occurred within 3 min, even with 20-fold less intestinal phosphatase than normally used (data not shown). The expression and phosphorylation of CREB isoform during BAT development was characterized by Western blotting. As shown in Fig. 4D, there was no significant change in the amount of CREB isoforms in total protein lysates; on the other hand, the levels of both CREBα and CREBΔ are more abundant in the nuclear extracts from 19-day fetus than those of 3-month-old mice. This result is consistent with the changes in the DNA-binding activity of the CRE and CRE2 sites (Fig. 1). Furthermore, by using a phospho-CREB-specific antibody, we found that the CREBΔ isoform, which is resistant to phosphatase treatment (Fig. 4C), is a major target for CREB phosphorylation in the nucleus at an early age; thereafter, CREB phosphorylation was shifted to CREBα isoform (Fig. 4D, bottom). Given that phosphatase-mediated dephosphorylation of CREB attenuates CREB activity (41, 42), changes in isoform-specific CREB phosphorylation may be a mechanism for CREB-associated regulation of gene expression during BAT development.

Increased Binding Activity of Adipogenic Transcription Factors during Cold Adaptation—The preceding analysis of transcription factor activity in interscapular brown adipose tissue during development of the mouse suggests that in the absence of a cold stimulus in adult mice, transcription factors associated with induction of thermogenesis are held in an inactive state by being sequestered in the cytoplasm. Because thermogenesis can be quickly induced in the adult mouse upon cold exposure, we determined the expression of these factors after this stimulus. To identify changes in adipogenic transcription factors in BAT during cold adaptation, binding activity of adipogenic transcription factors were measured by EMSA. Nuclear extracts were isolated from BAT of mice (male, 3-months old), maintained at 23 or 4°C for 1 and 7 days, and incubated with 32P-end-labeled probes, as described previously. Fig. 5 demonstrates that binding activity for PPARs, RXR, C/EBPs, and CREB increased upon cold exposure.

To determine the mechanism underlying these changes in DNA-binding activity of the transcription factors, their protein levels in total tissue extracts and nuclear extracts were measured by Western blot analysis. Levels of mRNA were determined by real-time RT-PCR. Consistent with previous reports, Ucp1 mRNA and protein levels increased after cold exposure (Fig. 5B); however, the changes observed in the expression of the transcription factors suggest a complex regulation. Pgc-1α mRNA was induced by cold exposure, but there was no induction in total protein extracts and only a small induction of PGC-1α protein in nuclear extracts. PPARγ1, -γ2, and PPARα were clearly induced at the protein level in both total-cell extracts and nuclear extracts, but there was no increase in mRNA levels (Fig. 5B). C/EBPβ and -δ protein was induced in both nuclear extracts and whole-cell extracts (Fig. 5C). Simi-
Fig. 3. Expression of the C/EBP during brown adipose tissue development. A, quantification of mRNA levels for C/EBPα ($\triangle$), C/EBPβ (●), and C/EBPδ (○) using real-time RT-PCR as described under “Materials and Methods.” B, Western blot analysis for C/EBPβ and C/EBPδ.

larly, induction of CREB isoforms occurred at the mRNA level and the protein level in the total-cell extracts and nuclear extracts (Fig. 5D). Thus, the reactivation of Ucp1 and other target genes of thermogenesis involved an increase in nuclear localization of transcription factors.

The total amount of PPARγ1 protein, the most dominantly expressed isoform in mature brown adipose tissue (Fig. 2B), was significantly increased after cold exposure, even though a slight decrease in PPARγ mRNA level was observed (Fig. 5B). It has been shown that nuclear hormone receptors, including PPARα and PPARγ, are targets for the ubiquitin-proteasome system of protein degradation (43–46). Because ligand activation has been shown to influence the stability of nuclear receptor proteins, the effect of norepinephrine on PPARγ was examined. A [35S]methionine pulse-chase experiment with differentiated brown adipocyte primary cultures showed that a significantly higher amount of [35S]-labeled PPARγ protein was retained in cells treated with 1 μM norepinephrine (Fig. 5E). This result suggests that increases in PPARγ protein after cold exposure of mice (Fig. 5B, top) are due, in part, to an increase in protein stability. In addition, there was no significant change in the mRNA level of PPARγ; however, the quantity of PPARα protein was slightly increased during cold exposure (Fig. 5B, left). Analysis of nuclear extracts by Western blotting (Fig. 5B, right) showed that the total amount of the PPARγ and PPARα in the nucleus increased after cold exposure, in parallel with the increased binding activity of a PPRE probe to PPARγ and PPARα proteins in nuclear extracts, as determined by EMSA (Fig. 5A).

PKA Expression and Nuclear Translocation—Given the important role that protein phosphorylation plays in the regulation of transcription factor activity and transport into the nucleus, the expression of PKA was determined in brown adipose tissue during development. Immunoblot analysis showed that the PKAα catalytic subunit was present in the nucleus, and its expression was not significantly altered during development (Fig. 6A). On the other hand, the PKA IIβ regulatory subunits were expressed at increased levels in nuclear extracts as mice aged (Fig. 6A). This was one of two proteins that showed an increased level of nuclear expression in aging mice; the other was phosphorylated PPAR (Fig. 4D). The results suggest that a reduced potential for phosphorylation of transcription factors occurs in the nucleus as mice reach maturity.

The effects of age on the nuclear localization of several transcription factors associated with adipogenesis suggest that a generalized down-regulation of transcriptional activity occurs in interscapular brown fat of the adult mouse and that PKA may have a major role in this process. Accordingly, we have looked at a transcription factor that could serve as an indicator of nuclear translocation. NF-κB translocation into the nucleus by PKA is a well-described mechanism and could serve this role, even though there is no evidence that NF-κB plays a role in the regulation of thermogenesis. However, it has recently been shown that NF-κB inhibits PPARγ-induced activation of adipogenesis in mesenchymal stem cells (47). We assessed DNA-binding activity and the level of NF-κB protein in nuclear protein extracts of brown adipose tissue during development. The latter included estimates of total p65 and its PKA-specific phosphorylation form by immunoblots. A large reduction in NF-κB DNA binding, immunoreactive total p65 and p65 phosphorylated on Ser276, the PKA-specific site, occurs during development. Importantly, this pattern of expression is partially reversed when adult mice are exposed to the cold (Fig. 6B). The evidence is consistent with reduced phosphorylation of p65 during development, which causes an accumulation of NF-κB in the cytoplasm with reduced transcriptional activity.

Nuclear translocation of PKAα catalytic subunit and NFκB in response to cold exposure and β-adrenergic stimulation was examined by immunoblot analysis and immunofluorescence cell staining. As shown in Fig. 7A, expression of PKAα catalytic subunit from nuclear extracts of brown adipose tissue increased after 1 and 7 days of cold exposure. Consistent with the immunoblot analysis, norepinephrine treatment increased nuclear staining of PKAα catalytic subunit in HIB-1B and pri-
Both HIB-1B and primary cultures from white adipose tissue showed cytoplasmic staining of the PKA catalytic subunit without β-adrenergic stimulation; however, primary cultures from brown adipose tissue showed significantly elevated nuclear staining for PKA subunit. Likewise, the nuclear localization of NF-κB p65 increased in response to norepinephrine treatment in HIB-1B and primary adipocyte cultures (Fig. 7C), which is consistent with the increase in DNA-binding activity and protein expression in nuclear extracts of brown adipose tissue from cold-exposed mice (Fig. 6B).

**DISCUSSION**

The evidence in this study suggests that sequestration of transcription factors in the cytoplasm is a major mechanism for reducing transcriptional activity in brown adipocytes of mice when the need for thermogenesis in adult mice is diminished.
need for thermogenesis is reduced, and transcription factors become increasingly sequestered in the cytoplasm where they are functionally inactive. The PPARs, RXRs, and C/EBPs function in brown fat to regulate both adipogenesis and thermogenesis. Consequently, this mechanism of sequestering transcription factors of adipogenesis into the cytoplasm may be unique to the brown adipocyte, because acute activation of thermogenesis does not require de novo adipogenesis. Chronic exposure of adult mice to the cold, which initiates cell proliferation, causes the translocation of transcription factors to the nucleus and, in addition, causes changes in mRNA and protein levels and new patterns of isoform expression.

**Fig. 5. Changes in adipogenic transcription factors in brown adipose tissue during cold adaptation.** A, binding activity of adipogenic transcription factors during cold adaptation. Autoradiogram of an EMSA using 32P-end-labeled probes (0.1 pmol) with nuclear extract (5 μg) from brown adipose tissue of 3-month-old mice exposed to the cold (4 °C). Methods and nucleotide sequences for probes are shown under “Materials and Methods.” B, top, Western blot analysis for PPARγ1 and -γ2, PPARα, PGC-1α, and UCP1. Mouse β-actin antibody was applied as a loading control. Bottom, quantification of mRNA levels for UCP1 (●), PPARα (□), PPARγ1 (○), PPARγ2 (●), and PGC-1α (■) using real-time RT-PCR. C, Western blot analyses for C/EBPβ and C/EBPδ as described under “Materials and Methods.” D, expression of the CREB isoforms during cold adaptation. Top, Western blot analysis for CREB. Bands are shown for CREBα, CREBβ, and CREBβ′ isoforms. Bottom, RT-PCR for quantification of CREB mRNA. cDNA from brown adipose tissue of B6 mice after cold exposure for indicated times were amplified using CREB isoform-specific primer pairs (see “Materials and Methods”) to evaluate expression of CREB isoforms during cold exposure. β-actin gene-specific primers were used for RT-PCR control. CREBα/β isoforms (335 bp) and CREBβ′ isoform (147 bp) are shown in agarose gel separation. E, autoradiogram of 32P-labeled PPARγ in a pulse-chase experiment with differentiated primary cultures from the stromal vascular fraction from brown adipose tissue. See “Materials and Methods” for experimental details.
Eukaryotic organisms from yeast to mammals, the sequestration of transcription factors in the cytoplasm has been proposed as an important mechanism for the control of gene expression during development and the metabolism of selected tissues in response to environmental factors. Proteins that are imported into the nucleus require a nuclear localization signal (NLS) for passage through a nuclear pore complex in the nuclear membrane. Regulation of the movement of proteins with an NLS to the nucleus is controlled by a combination of phosphorylation of sequences flanking the NLS and masking of the NLS by cytoplasmic retention proteins. As shown for PKC-mediated phosphorylation of lamin B2, phosphorylation of an NLS region can either inhibit or stimulate import into the nucleus, as evidenced by casein kinase II phosphorylation of nucleoplasmin. In addition, a combination of protein phosphorylation and interaction with cytoplasmic retention proteins are involved in the import mechanism of some proteins, such as NF-κB, for which a well characterized PKA-
mediated mechanism has been described for nuclear import and intranuclear interactions with the transcriptional coactivator CREB-binding protein (53–55). The presence of nuclear localization signals on adipogenic transcription factors have been described in some detail for C/EBP (56) and CREB (57), and they have been inferred from large deletions for PGC-1α (58) and a mutation forming a truncated PPARα (59); however, there have been no reports indicating that a change in nuclear localization is a mechanism for regulating adipogenesis. In addition, phosphorylation is a common mechanism associated with the regulation of nuclear translocation, and although phosphorylation occurs on adipogenic transcription factors (58, 60, 61), the function of phosphorylation in the control of adipogenesis is not well understood (62), nor has it been previously associated with mechanisms of nuclear translocation.

The mouse is born with functional brown adipose tissue to maintain body temperature upon the abrupt reduction in ambient temperature experienced at birth (1). We know from the cold sensitivity of mice deficient in norepinephrine that adrenergic signaling is required for the activation of thermogenesis (5). This process is mediated by the β-adrenergic receptors which activate the cAMP-dependent protein kinase A pathway and the downstream transcriptional mechanisms that increase Ucp1 expression and mitochondrial biogenesis (63–66). We have presented evidence in this paper showing that the cytoplasmic to nuclear distribution of transcription factors associated with Ucp1 expression could be a key step in the control of thermogenesis. Given that a mechanism has been well described by which the PKA signaling pathway participates in the control of the nuclear translocation of NF-xB (67), we have used NF-xB nuclear translocation as an indicator for PKA-dependent mechanisms of transcription factor translocation in brown adipose tissue during development. NF-xB has recently been shown to inhibit the ability of PPARγ to induce adipogenesis in a mesenchymal stem cell model (47); therefore, it was surprising to find that an NF-xB probe binding in nuclear extracts from brown adipose tissue was reduced in parallel with adipogenic transcription factors (Fig. 6B). This indicates that NF-xB is sequestered in the cytoplasm in a manner similar to adipogenic transcription factors and that nuclear relocalization is stimulated by cold exposure. Analysis of the phosphorylation patterns of the p65 subunit indicates that it is drastically reduced in the nucleus of older animals and suggests a mechanism for cytoplasmic sequestration dependent upon down-regulation of PKA activity. To evaluate further the effect of adrenergic stimulation upon the increase in nuclear localization of NF-xB, we showed the enhanced presence of nuclear NF-xB in the nucleus of primary adipocytes by immunofluorescence (Fig. 7C). The advantage to the organism of a mechanism for controlling the expression of thermogenic genes by phosphorylation-dependent nuclear translocation is that the transcription machinery is already present and, therefore, able to respond quickly to signals for the activation of thermogenesis.

Our results suggest that a coordinated mechanism controls the cytoplasmic retention of both leucine zipper and helix-loop-helix transcription factors during development. Studies on the cytoplasmic retention of transcription factors have generally described a mechanism for the regulation of a single factor (48). A common mechanism that has been well documented for the control of nuclear import has been the role of phosphorylation by several kinases, including PKA, casein kinase II, and cdk kinase. The SV40 T-antigen has two phosphorylation sites near the NLS: one accelerates nuclear import and the other reduces nuclear import (48). Phosphorylation has also been shown to be an important regulator of C/EBPβ, PPARα (68), PPARγ (69), RXR (61), and PGC-1α during cytokine stimulation of energy expenditure (70). Given that thermogenesis in brown adipose tissue is initiated by the interaction of catecholamines with cell-surface adrenergic receptors that activate a cAMP-dependent protein kinase A phosphorylation pathway, a mechanism involving phosphorylation for the regulation of thermogenic transcription is plausible. However, the effects of phosphorylation on the adipogenic transcriptional activity are generally on DNA binding or ligand activation.

Although the major change occurring during development seems to be the cytoplasmic sequestration of transcription factors, with little reduction in total mRNA or protein, reactivation of thermogenesis in the adult mouse initiates translocation of factors into the nucleus as well as changes in mRNA and protein levels and changes in isoform expression for CREB and PPARs. Because cold exposure of mice causes an acute thermogenic response followed by a chronic response, which involves de novo proliferation of brown adipocytes, the adipogenesis program must also be activated. Most interesting is the observation that PPARγ protein levels are induced despite the absence of detectable induction of mRNA, which is consistent with previous observations of PPARγ mRNA expression in rat BAT (71). Several nuclear hormone receptors, such as RXRα/γ, RARα, thyroid hormone receptor, and PPARγ, are degraded by the ubiquitin-proteasome system (43–45). Recently, Blanquart et al. (46) have shown that the protein stability of PPARα is increased in the presence of its ligand WY 14,643 by decreasing its ubiquitination. Consistent with these reports, we show with a pulse-chase experiment that the stability of PPARγ increases in differentiated brown adipocyte cultures after treatment with norepinephrine in a manner similar to that caused by proteasome inhibitors. This result suggests an additional mechanism for the control of transcription during thermogenesis.

In addition to proposing that a mechanism by which transcription factors are sequestered into a cytoplasmic compartment in thermogenically quiescent tissue, we have shown that changes occur in isoform expression for CREB and PPAR. Alternative exon slicing generates isoforms of CREB that vary functionally, depending upon their domain composition. These are the centrally located kinase inducible domain, which is phosphorylated at Ser133 by protein kinase A activation, hydrophobic glutamine-rich domains that function as constitutive transcriptional activators (Q1 and Q2/CAD), the carboxyl-terminal basic leucine zipper domain for the dimerization with all CREB family members, as well as functionally unknown α/γ domains in the middle of the protein. It has been demonstrated that targeting of the CREB gene leads to up-regulation of a CREBβ isoform which is missing the glutamine-rich domain (Q1) and α/γ domain because of an increase in alternative splicing or mRNA stability (39). In this study, we have shown that the CREBα isoform, which contains α domains between glutamine-rich transactivation domain (Q1) and the kinase-inducible domain, as well as CREBβ isoforms, are resistant to phosphatase-mediated dephosphorylation. We found that CREBα is a major target for CREB phosphorylation during fetal development and early post-natal life. On the other hand, expression of CREBβ was increased in brown adipose tissue after cold exposure in adult mice. It has been shown that CREB dephosphorylation by serine/threonine phosphatase, PP-1 (41) and PP-2A (42) is a mechanism to attenuate CREB-mediated transcription in the PKA-dependent signaling pathway. These results imply that selective phosphorylation of the CREBα or -γ domains and exon splicing might be an important mechanism to regulate CREB activity through phosphatase-mediated CREB dephosphorylation in brown adipose tissue. Further study will be necessary to address isoform-specific CREB phos-
phorylation and its association with BAT development and Ucp1 induction. In summary, the changing requirements for thermogenesis in the mouse, from the late fetus to the adult stage, are accompanied by changes in the transcriptional machinery that include sequestration of nuclear transcripational factors into the cytoplasm. Changes also occur in the distribution of PKA regulatory subunits and phosphorylation and its association with BAT development and Ucp1 induction.

The observed changes in PKA regulatory subunits and phosphorylation in the control of brown adipose tissue thermogenesis suggest that phosphorylation plays a key role in the control of transcription and nuclear localization during thermogenesis. The observed changes in NF-κB DNA-binding activity and nuclear localization are consistent with this model.

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