Depletion of cAMP-response Element-binding Protein/ATF1 Inhibits Adipogenic Conversion of 3T3-L1 Cells Ectopically Expressing CCAAT/Enhancer-binding Protein (C/EBP) α, C/EBP β, or PPARγ2

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The differentiation of preadipocytes to adipocytes is orchestrated by the expression of the “master adipogenic regulators,” CCAAT/enhancer-binding protein (C/EBP) β, peroxisome proliferator-activated receptor γ (PPARγ), and C/EBP α. In addition, activation of the cAMP-response element-binding protein (CREB) is necessary and sufficient to promote adipogenic conversion and prevent apoptosis of mature adipocytes. In this report we used small interfering RNA to deplete CREB and the closely related factor ATF1 to explore the ability of the master adipogenic regulators to promote adipogenesis in the absence of CREB and probe the function of CREB in late stages of adipogenesis. Loss of CREB/ATF1 blocked adipogenic conversion of 3T3-L1 cells in culture or 3T3-F442A cells implanted into athymic mice. Loss of CREB/ATF1 prevented the expression of PPARγ, C/EBP α, and adiponectin and inhibited the loss of Pref-1. Loss of CREB/ATF1 inhibited adipogenic conversion even in cells ectopically expressing C/EBP α, C/EBP β, or PPARγ2 individually. CREB/ATF1 depletion did not attenuate lipid accumulation in cells expressing both PPARγ2 and C/EBP α, but adiponectin expression was severely diminished. Conversely ectopic expression of constitutively active CREB overcame the blockade of adipogenesis due to depletion of C/EBP β but not due to loss of PPARγ2 or C/EBP α. Depletion of CREB/ATF1 did not suppress the expression of C/EBP β as we had previously observed using dominant negative forms of CREB. Finally results are presented showing that CREB promotes PPARγ2 gene transcription. The results indicate that CREB and ATF1 play a central role in adipogenesis because expression of individual master adipogenic regulators is unable to compensate for their loss. The data also indicate that CREB not only functions during the initiation of adipogenic conversion but also at later stages.

Normal adipose tissue development and increases in adipose tissue mass associated with weight gain and obesity require the formation of mature adipocytes from preadipocytes or stromal progenitor cells (1–3). The factors and processes that mediate the conversion of preadipocytes to mature adipocytes have been elucidated, at least in part, in immortalized preadipocyte cell lines like 3T3-L1, 3T3-F442A, and Ob1771 and in primary preadipocytes and stromal progenitor cells (1, 5–8). Adipogenic conversion is initiated in culture via the addition of glucocorticoids and insulin or insulin-like growth factor-1 plus agents that elevate intracellular cAMP levels. Ligands of the nuclear hormone receptor, PPARγ2 like thiazolidinediones and prostaglandin, also increase the rate or extent of adipogenesis (9, 10). The agents that govern adipogenic conversion in vivo are less well understood, but there is convincing evidence that free fatty acids, insulin and insulin-like growth factor-1, and glucocorticoids play important roles in the development of adipose tissue and the formation of new adipocytes.

Exposure to differentiation-inducing agents initiates a well characterized cascade of gene expression events that lead to the mature adipocyte phenotype. The differentiation cascade begins with the expression of C/EBP β within hours of treatment with inducing agents (11). This factor then promotes the expression of PPARγ and C/EBP α (12, 13). Both of these factors are necessary to promote the terminal or mature adipocyte phenotype including insulin-sensitive glucose uptake (14). However, all of these “master adipogenic regulators” are expressed after the initiation of the differentiation program. This suggests that pre-existing regulatory factor(s) are required to initiate adipogenic conversion.

In previous studies we demonstrated that the transcription factor CREB was expressed in 3T3-L1 preadipocytes before treatment with differentiation-inducing agents, in mature 3T3-L1 adipocytes, and throughout the adipogenic cascade (15, 16). In addition, we showed that CREB phosphorylation and

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1The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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2 The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; CREB, cAMP-response element-binding protein; C/EBP, CCAAT/enhancer-binding protein; siRNA, small interfering RNA; CREM, cAMP-response element modulator; ERK, extracellular signal-regulated kinase; CRE, cAMP-response element; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; Bt,cAMP, dibutyryl cyclic AMP; PVDF, polyvinylidene difluoride; GFP, green fluorescent protein.
transcriptional activity were activated by both cAMP mimetics via protein kinase A and by insulin through the ERK signaling pathway. Subsequently we demonstrated that ectopic expression of constitutively active forms of CREB was sufficient to drive adipogenic conversion of 3T3-L1 cells, whereas dominant negative forms of CREB inhibited this process (16). The central role of CREB in adipogenesis is exemplified by the ability of constitutively active forms of CREB to overcome inhibition of adipogenic conversion due to blockade of Ras/ERK signaling (17). In addition, diminished CREB activity in mature adipocytes induces apoptosis, in part, through decreased Akt expression (18). More recently, in collaboration with Lane and co-workers (19), we have reported evidence that CREB promotes adipocytokine secretion (18). The results indicate that CREB and ATF1 play a central role in adipogenesis because expression of individual master adipogenic regulators is unable to compensate for their loss. The data also indicate that CREB not only functions during the initiation of adipogenic conversion but at later stages as well.

### EXPERIMENTAL PROCEDURES

**Materials**—Cell culture media and supplies were from Invitrogen, Gemini Bioproducts (Gaithersburg, MD), and Specialty Media, Inc. (Lavallette, NJ). 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA), and 3T3-F442A cells were provided by Dr. Stephen Farmer (Boston University, Boston, MA). Expression vectors for the dominant negative CREB inhibitor protein KCREB (pRSV-KCREB) and the constitutively active CREB isoform CREB-DIEDML (pRSV-CREB-DIEDML) were provided by Dr. Richard Goodman (Oregon Health Sciences University, Portland, OR). A vector containing the proximal C/EBP β gene promoter from -121 to +16 (pLAPPRO 8) or a promoter in which the two CRE sites were ablated (pLAPPRO 8 I) (20) were obtained from Dr. Christian Trautwein (Medizinische Hochschule Hannover, Hannover, Germany). Plasmids for stable expression of PPARγ2 (pTS13-PPARγ2) and C/EBP α (pLXSN-C/EBP α) were provided by Mitch Lazar (University of Pennsylvania, Philadelphia, PA). Plasmids for stable expression of C/EBP β (pWZL-C/EBP β) and LacZ (pWZL-LacZ) were provided by Dr. Robert Lewis (University of Nebraska, Omaha, NE). Plasmids for stable siRNA expression, pSilencer 4.1-CMVneo and pSilencer 4.1-CMVpuro, were purchased from Ambion, Inc. (Austin, TX). The double-stranded siRNA oligonucleotides listed in Table 1 were purchased from Dharmacon, Inc. (Lafayette, CO), and the double-stranded oligonucleotides used to make stable siRNA expression plasmids were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Rabbit polyclonal antibodies for CREB and C/EBP β were purchased from Cell Signaling Technology (Danvers, MA), and a polyclonal antibody to Pref-1 was obtained from Alpha Diagnostics International (San Antonio, TX). Polyclonal antibodies to CREM, ATF1, ATF2, c-Jun, and C/EBP α and a

### Table 1

#### Oligonucleotides

| Target | Plasmid | Sense strand sequence |
|--------|---------|-----------------------|
| siRNA oligonucleotides | Luciferase (control) | pSilencer 4.1-CMVneo 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |
| CREB | pSilencer 4.1-CMVneo | 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |
| CREM | pSilencer 4.1-CMVpuro | 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |

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#### Oligonucleotides for stable siRNA expression

| Target | Plasmid | Sense strand sequence |
|--------|---------|-----------------------|
| Luciferase (control) | pSilencer 4.1-CMVneo | 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |
| CREB | pSilencer 4.1-CMVneo | 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |
| CREM | pSilencer 4.1-CMVpuro | 5'-ACC TTC TCC ACG TAC GGA TAG CTC TTG AAT CTA AGT ATT CCG CTT ACC AGG G-3' |
CREB Loss Inhibits Adipogenesis

Monoclonal antibody to PPARγ2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Luciferase assay reagents and the plasmid pRL-TK, containing the enhancerless thymidine kinase gene promoter driving a Renilla luciferase reporter gene, were obtained from Promega Corp. (Madison, WI). SuperSignal West Pico chemiluminescent substrate was from Pierce. Horseradish peroxidase-conjugated secondary antibodies were obtained from Vector Laboratories (Burlingame, CA). Oligofectamine, Lipofectamine, and PLUS reagent were obtained from Invitrogen. ApoAlert DNA fragmentation (TUNEL) kits were obtained from BD Biosciences. Low growth factor Matrigel was obtained from BD Biosciences.

**Cell Culture and Adipocyte Differentiation in Vitro**—3T3-L1 and 3T3-F442A preadipocytes were passaged in low glucose Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum (FCS), and 1 mM l-glutamine. 3T3-L1 fibroblasts were differentiated into adipocytes after siRNA and/or plasmid transfection upon reaching confluency by the addition of high glucose DMEM containing 10% FCS, 1 mM l-glutamine, and 300 μM isobutylmethylxanthine or β,γ-cAMP, 1 μM dexamethasone, and 1 μg/ml insulin (MDI). After 2 days, the 3T3-L1 cells were transferred to high glucose DMEM plus 10% FCS, 1 mM l-glutamine, and 1 μg/ml insulin and refed every 2 days. Differentiation of preadipocytes to mature adipocytes was confirmed by Oil Red O staining of lipid vesicles.

**Western Blotting**—Whole cell lysates were prepared in 20 mM Tris-HCl containing 10% glycerol, 0.3% Nonidet P-40, 300 mM NaCl, 1.5 mM Mg2Cl, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (Complete mini protease inhibitor tablets, Roche Applied Science). After correcting for protein concentrations, cell lysates were mixed with an equal volume of Laemmli SDS loading buffer, and equal amounts of lysate protein were resolved on 10% polyacrylamide-SDS gels and transferred to PVDF membranes. The blots were blocked with phosphate-buffered saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize the target proteins indicated in each figure overnight at 4 °C. The blots were washed and subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase. After the blots were washed, specific immune complexes were visualized with SuperSignal West Pico chemiluminescent substrates.

**siRNA and Plasmid Transfection and Luciferase Assays**—For siRNA transfection, 3T3-L1 cells were plated at 30–50% confluency on 6-well plates in complete medium. Twenty-four hours later the cells were transferred to Opti-MEM for transfection. Double-stranded siRNA oligonucleotides or the control luciferase-specific siRNA was complexed with Oligofectamine reagent and applied to the cells according to the manufacturer’s recommendations at a final concentration of 200 nM. Where more than one siRNA oligonucleotide is specified in Table 1, equimolar amounts of the individual oligonucleotides were combined before use. After 3 h, an equal volume of DMEM containing 30% FCS was added to the wells. Cells were allowed to recover for 24 h before subsequent manipulations.

For plasmid transfections, plates of 3T3-L1 or 3T3-F442A preadipocytes were grown to 70–80% confluency and transfected with the indicated plasmids with Lipofectamine PLUS reagents according to the manufacturer’s recommendations. Cells transiently transfected with the C/EBP β gene promoter-luciferase or PPARγ2 gene promoter-luciferase reporter plasmids were used within 48 h of transfection. Cells stably transfected with the PPARγ2 expression plasmid were selected in hygromycin (100 μg/ml). Cells stably expressing C/EBP β and LacZ expression vectors were selected in blasticidin (10 μg/ml), whereas cells transfected with the PPARγ2 expression plasmid were selected in hygromycin (100 μg/ml). Cells stably expressing C/EBP β and LacZ expression vectors were selected in blasticidin (10 μg/ml). Large, rapidly growing, well separated colonies were isolated 10–12 days after selection was begun with the antibiotics. Isolated clones were passaged in low glucose DMEM containing 10% FCS and 1 mM l-glutamine with half the concentration of selection antibiotic used during selection.

Luciferase assays were conducted on lysates from cells transfected with plasmids containing the C/EBP β gene promoter or the PPARγ2 gene promoter (~611 to +32) linked to the firefly luciferase gene. Cells were co-transfected with the plasmid pRL-TK as an internal control. Cell lysates and luciferase reactions were performed with the Dual-Luciferase reporter assay system on a Turner Designs 20/20® luminometer (Turner Designs, Sunnyvale, CA).

**Microscopy**—Microscopy was performed on a Nikon TE2000-U inverted epifluorescence microscope. Phase-contrast, bright field images were captured to a personal computer with a Spot Insight color camera (Diagnostic Imaging, Sterling Heights, MI). Images were analyzed and processed with MetaMorph 6.1 Software (Molecular Devices, Sunnyvale, CA).

**Apoptosis Assays**—TUNEL staining was performed using ApoAlert DNA fragmentation assay kits according to the manufacturer’s directions.

**Stable Expression of Control and CREB siRNAs**—Stable expression of siRNAs specific for luciferase or CREB was achieved by ligating the double-stranded DNA oligonucleotides specified in Table 1 into pSilencer 4.1 vectors. The luciferase oligonucleotide was inserted into pSilencer 4.1-CMVneo. A double-stranded oligonucleotide corresponding to CREB siRNA oligo B was inserted into pSilencer 4.1-CMVneo, and another corresponding to CREB siRNA oligo C was inserted into pSilencer 4.1-CMVpuro.

The plasmids were transfected into 3T3-F442A cells as described above. Cells for stable depletion of CREB were transfected sequentially with both CREB siRNA expression plasmids and selected in both G418 (1 mg/ml) and puromycin (2 μg/ml). Cells transfected with the luciferase siRNA expression plasmid were selected in G418 alone. Large, rapidly growing colonies were isolated and pooled for implantation into athymic mice as described below.

**In Vivo Adipogenesis in Matrigel**—3T3-F442A preadipocytes were stably transfected with plasmids for the constitutive expression of siRNA for luciferase (control) or CREB as described above. Cells were selected in appropriate antibiotic until large, rapidly growing colonies were present. Colonies were mixed and grown to 50–70% confluence on 10-cm plates. The cells were then infected with an adenovirus expressing green fluorescent protein (GFP) at a multiplicity of infection of 100. The next day, cells were trypsinized and recovered by centrifugation. The pellets (~2 × 10⁶ cells) were gently resuspended in 200 μl of low growth factor Matrigel at 4 °C.

**In Vivo Adipogenesis in Matrigel**—3T3-F442A preadipocytes were stably transfected with plasmids for the constitutive expression of siRNA for luciferase (control) or CREB as described above. Cells were selected in appropriate antibiotic until large, rapidly growing colonies were present. Colonies were mixed and grown to 50–70% confluence on 10-cm plates. The cells were then infected with an adenovirus expressing green fluorescent protein (GFP) at a multiplicity of infection of 100. The next day, cells were trypsinized and recovered by centrifugation. The pellets (~2 × 10⁶ cells) were gently resuspended in 200 μl of low growth factor Matrigel at 4 °C.
Female athymic mice were lightly anesthetized with Halothane, and the Matrigel/cell suspensions were injected subcutaneously into the abdomen anterior of the thigh. Fresh food, water, and clean cages with fresh bedding were provided every other day for a period of 2 or 4 weeks. Light was maintained on a 12-h cycle, and humidity was 40–45% with a temperature of 25–27 °C. The animals were monitored daily, and weight was checked once a week. The mice were then anesthetized with Halothane and euthanized by exsanguinations. The solid Matrigel plugs and adjacent skin and subcutaneous muscle were removed. The plugs were fixed overnight in 4% paraformaldehyde and then sliced in half with a razor blade. The halves were oriented in paraffin blocks with the cut surfaces up for sectioning.

Five-micrometer sections were deparaffinized in HemoD and rehydrated in graded ethanol series. The sections were stained with hematoxylin and eosin for histological examination. Alternately deparaffinized sections were mounted with Permount for visualization of GFP.

**Chromatin Immunoprecipitation for CREB Promoter Binding**—Chromatin immunoprecipitation assays for CREB binding to the PPARγ2 gene promoter were performed on 3T3-L1 cells treated with MDI for 48 h using kits from Upstate (Charlottesville, VA) according to their directions. The procedure was modified as described previously (19).

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays and supershift assays were used to assess binding of CREB, ATF2, c-Jun, and CREM to the distal C/EBP gene promoter CRE site. A double-stranded DNA oligonucleotide corresponding to the promoter from −117 to −96 with respect to the transcription start was used in the reactions, which were performed as described previously (19).

**RESULTS**

**Depletion of CREB/ATF1 Blocks 3T3-L1 Cell Adipogenesis in Culture**—We previously reported that ectopic expression of constitutively active forms of CREB alone is sufficient to drive adipogenesis. Alternately ectopic expression of dominant negative forms of CREB blocked adipogenic conversion of 3T3-L1 cells treated with differentiation-inducing agents. To further explore the impact of CREB on adipocyte differentiation, we used specific siRNA as a means of depleting CREB in 3T3-L1 cells. We found that CREB-specific siRNA reduced CREB levels in 3T3-L1 preadipocytes to almost undetectable levels within 72 h of transfection (Fig. 1A). Although CREB levels began to increase 6 days after siRNA transfection, they never achieved levels measured in untreated cells or cells treated with control siRNA. CREB-specific siRNA did not affect the expression of other CRE-binding factors including ATF2, CREM (Fig. 1A), or c-Jun (data not shown).

**FIGURE 1.** Depletion of CREB and ATF1 inhibits adipogenic conversion of 3T3-L1 cells. 3T3-L1 preadipocytes were transfected with siRNA for luciferase as a control (Cntrl) or CREB/ATF1 (equimolar mixture of three double-stranded oligonucleotides listed in Table 1) as described under “Experimental Procedures.” The cells were then replated on 24-well plates to bring the cells to confluence and growth arrest. The cells were then transfected with siRNA. A, on the days shown at the top of the figure, cells were harvested and lysed. Twenty-five micrograms of lysate protein was resolved on 10% polyacrylamide-SDS gels and transferred to PVDF membranes. The blots were blocked and probed with antibodies to the factors indicated to the left of each panel. The blots were washed and incubated with horseradish peroxidase-linked secondary antibodies, and the specific immune complexes were visualized with SuperSignal West Pico reagents. B, representative phase-contrast photomicrographs of 3T3-L1 cells on day 9 of the treatment regimen. Cells were stained with Oil Red O to visualize lipid vesicles. LAP, liver enriched activator protein; LIP, liver enriched inhibitory protein.
Cells transfected with control or CREB-specific siRNA were then treated with the differentiation-inducing mixture, MDI, to evaluate the loss of CREB and ATF1 on adipogenesis. PPARγ2 and C/EBPα expression were detected in control siRNA-treated cells beginning on day 3 after MDI exposure (Fig. 1A). PPARγ2 levels continued to increase over the 9-day differentiation period, whereas levels of C/EBPα peaked at day 6 and then declined slightly at day 9. The expression of these factors was delayed and the levels were much lower in CREB siRNA-treated cells. Levels of Pref-1, an inhibitor of adipogenesis, declined rapidly in cells transfected with control siRNA and treated with MDI. Pref-1 levels were elevated in CREB siRNA-treated cells before MDI exposure and remained elevated for at least 72 h after addition of these agents. Thereafter Pref-1 levels declined but were still detectable 6 days after MDI exposure. Unexpectedly loss of CREB/ATF1 had little impact on C/EBPβ liver enriched activator protein or liver enriched inhibitory protein expression.

The ability of CREB siRNA to inhibit adipogenic conversion was also apparent in Oil Red O-stained cell preparations. Although control siRNA-treated cells accumulated substantial levels of triglyceride, only minute lipid droplets were present in a small percentage of cells transfected with CREB siRNA (Fig. 1B). These data indicate that CREB and/or ATF1 is required for MDI-induced adipogenesis in culture.

The expression of other CRE-binding proteins like ATF2 and c-Jun or their activation by extracellular signals is increased during the early stages of adipogenesis (21, 22). Likewise CREM, another CREB family member, is expressed in preadipocytes (19). To determine whether these factors were required for adipogenic conversion, they were individually depleted via siRNA. Depletion of ATF2, c-Jun, and CREM was verified by Western blot assay (Fig. 2A). We found that loss of these factors had no significant effect on the conversion of preadipocytes to mature, lipid-filled adipocytes (Fig. 2B).

Depletion of CREB/ATF1 Blocks 3T3-F442A Cell Adipogenesis in Vivo—Although the role of CREB in adipocyte differentiation and survival has been examined in cultured cells, its role in in vivo adipogenesis has not been explored. To begin to address this issue, we generated stably transfected 3T3-F442A preadipocytes constitutively expressing either control or CREB-specific siRNAs. These cells were implanted subcutaneously into athymic mice in low growth factor Matrigel plugs for a period of 2 or 4 weeks. During this time the plugs become vascularized such that proadipogenic factors produced by the recipient mice promote adipogenic conversion of the implanted cells. Twenty-four hours before their implantation, the cells were infected with an adenovirus from which GFP was constitutively expressed to follow their fate.

As shown in Fig. 3A, cells expressing control siRNA formed substantial numbers of unilocular and multilocular adipocytes within the Matrigel plug 2 weeks after implantation. The number of adipocytes and the size of the lipid droplets increased slightly at the 4-week time point. That these adipocytes arose from the implanted cells is evident by their GFP fluorescence (Fig. 3B). However, cells expressing CREB siRNA failed to form adipocytes at either time point (Fig. 3A). No significant differences in cell survival were detected by TUNEL staining of the plug sections (data not shown). These results indicate that the role of CREB in adipogenesis extends to the in vivo formation of adipocytes.

Depletion of CREB/ATF1 Blocks Adipogenesis of 3T3-L1 Cells Expressing C/EBPα, C/EBPβ, or PPARγ—Ectopic overexpression of C/EBPα or β or PPARγ alone is sufficient to promote adipogenic conversion and increase or accelerate adipogenesis in cells treated with MDI (8). The goal of subsequent experiments was to determine whether loss of CREB/ATF1 could block the proadipogenic activities of these master regulators. We generated stably transfected 3T3-L1 cells constitutively expressing C/EBPβ, C/EBPα, or PPARγ2. These cells were then transfected with control or CREB-specific siRNA and then left untreated or treated with MDI. Adipogenic conversion of these cells was measured by the appearance of Oil Red O-staining lipid droplets and by the appearance of adipocyte markers.

As shown previously, CREB siRNA blocked MDI-induced lipid accumulation and prevented the modest lipid accumulation detected in control siRNA-transfected cells in the absence of MDI (Fig. 4A). In cells expressing C/EBPα or β or PPARγ and transfected with control siRNA, a small percentage of cells exhibited substantial lipid accumulation even in the absence of MDI. With MDI, these cells showed normal or slightly higher
than normal levels of lipid content. Cells expressing both PPARγ2 and C/EBP α and treated with control siRNA exhibited substantial lipid accumulation regardless of MDI exposure. However, lipid droplets were greatly diminished in CREB/ATF1-depleted cells expressing C/EBP β or PPARγ2 with or without MDI. Loss of CREB/ATF1 also reduced uninduced or MDI-induced lipid accumulation in C/EBP α-expressing cells but not to the extent seen in the C/EBP β- or PPARγ2-expressing cells. Interestingly, CREB/ATF1 depletion had no effect on lipid storage in cells expressing both C/EBP α and PPARγ2, suggesting that combined expression of these factors can overcome the blockade of adipogenesis due to CREB/ATF1 loss.

These results were largely confirmed by Western blotting for C/EBPs α and β, PPARγ2, and the mature adipocyte marker adiponectin (23) (Fig. 4B). In control cells expressing LacZ, loss of CREB/ATF1 blocked C/EBP α and PPARγ2 expression and significantly blunted adiponectin expression in untreated or MDI-treated cells. However, CREB/ATF1 depletion had no effect on C/EBP β expression in confirmation of the results shown in Fig. 1. In cells constitutively expressing C/EBP β and transfected with control siRNA, PPARγ2, C/EBP α, and adiponectin were increased with MDI and were slightly elevated in MDI-naive cells compared with the LacZ-expressing cells. Loss of CREB/ATF1 in these cells lowered basal PPARγ2, C/EBP α, and adiponectin levels and blunted the ability of MDI to increase their expression. Once again, CREB/ATF1 depletion had no effect on C/EBP β levels in these cells under unstimulated or MDI-treated conditions.

Ectopic expression of PPARγ2 led to a substantial increase in C/EBP α levels in cells transfected with control siRNA with or without MDI. PPARγ2 expression also produced a modest increase in adiponectin in MDI-naive cells and further stimulated adiponectin levels in MDI-treated cells. However, CREB/ATF1 depletion repressed both C/EBP α and adiponectin expression in both untreated and treated cells. No change in C/EBP β levels was noted in these cells.

Forced expression of C/EBP α in cells transfected with control siRNA had little impact on basal or MDI-stimulated C/EBP β levels. Ectopic C/EBP α expression produced a mild increase in basal PPARγ2 and adiponectin expression, consistent with
the modest increase in Oil Red O staining observed in these cells. Interestingly, CREB siRNA diminished both basal and MDI-stimulated PPARγ2 and adiponectin expression but again had no effect on C/EBP β levels. These data confirm that loss of CREB/ATF1 blocks adipogenesis and acquisition of the mature adipocyte phenotype despite the expression of the master adipogenic regulators.

Finally combined expression of PPARγ2 and C/EBP α had no effect on C/EBP β expression in control or CREB-depleted cells. MDI stimulated adiponectin expression in untreated cells transfected with control siRNA, but low levels were detected in MDI-naive cells. However, adiponectin was not detected in MDI-naive cells depleted of CREB/ATF1, and levels were greatly diminished even in MDI-treated cells. Thus, although combined expression of PPARγ2 and C/EBP α promotes lipid accumulation in the absence of CREB/ATF1, other markers of the mature adipocyte phenotype are not rescued.

**Activation of CREB Overcomes Inhibition of Adipogenesis due to Loss of C/EBP β but Not due to Loss of PPARγ2 or C/EBP α**—We previously demonstrated that ectopic expression of constitutively active forms of CREB stimulated adipogenesis in 3T3-L1 cells (16) even when the ERK signaling pathway was repressed (17). In the current studies, we next sought to determine whether increased CREB activity would stimulate adipogenic conversion in cells depleted of C/EBP α or β or PPARγ2.

In cells treated with control siRNA, expression of CREB-DIEDML alone increased C/EBP β, PPARγ2, C/EBP α, and adiponectin to levels comparable to those observed in cells treated with MDI (Fig. 5B). CREB-DIEDML also stimulated lipid accumulation but to a lesser extent than MDI (Fig. 5A).

In cells depleted of C/EBP β with siRNA, MDI only modestly increased lipid storage (Fig. 5A) and failed to elicit a robust increase in PPARγ2, C/EBP α, or adiponectin (Fig. 5B). Conversely CREB-DIEDML alone increased lipid accumulation in C/EBP β-deficient cells albeit to a lesser extent than in cells treated with control siRNA. PPARγ2, C/EBP α, and adiponectin expression was elevated by constitutively active CREB in C/EBP β-depleted cells.

Loss of PPARγ2 effectively blocked MDI-induced lipid storage (Fig. 5A) and C/EBP β and adiponectin expression but had no effect on the induction of C/EBP β (Fig. 5B). CREB-DIEDML did not significantly increase lipid accumulation and was unable to stimulate C/EBP α or adiponectin expression in these cells.

Finally depletion of C/EBP α did not inhibit MDI induction of C/EBP β or PPARγ2 but did prevent adiponectin expression (Fig. 5B) and lipid storage (Fig. 5A) in MDI-treated cells. Like MDI, CREB-DIEDML stimulated both C/EBP β and PPARγ2 expression but did not restore adiponectin expression in C/EBP α-deficient cells. CREB-DIEDML also failed to increase lipid accumulation in these cells. These results show that activation of CREB alone promotes adipogenesis even in cells lacking C/EBP β. However, constitutively active CREB cannot overcome the loss of PPARγ2 or C/EBP α in the adipogenic program.

**siRNA Does Not Affect 3T3-L1 Cell Survival**—Transfection of 3T3-L1 cells with siRNA could potentially regulate adipogenesis by mechanisms aside from its impact on target gene expression. The effect of each individual siRNA used in these studies on cell survival was investigated by TUNEL staining for apoptotic nuclei. Supplemental Fig. 1 shows that none of the siRNAs significantly increased the percentage of apoptotic cells 72 h post-transfection. Similar data were obtained 6 and 9 days post-siRNA transfection (not shown).

**C/EBP β Expression Is Regulated by CREB/ATF1, ATF2, c-Jun, and CREM**—We and other groups have demonstrated that expression of the C/EBP β gene is mediated by two adjacent CRE sites in the C/EBP β gene promoter (19, 20). This was confirmed by experiments in which basal and MDI-stimulated transcription was measured from the wild type C/EBP β gene promoter and a mutant promoter in which the two CREs were ablated (∆CRE2x). Transcription from the wild type promoter was stimulated almost 4-fold by MDI, but both basal and MDI-induced levels were severely diminished with the mutant promoter (Fig. 6A).

We have also reported that inhibition of CREB DNA binding activity with the dominant negative factors ACREB or KCREB repressed basal and cAMP-stimulated transcription from the C/EBP β gene promoter (19). This response was confirmed in co-transfection studies in which transcription from the wild type C/EBP β gene promoter was measured in cells expressing the LacZ gene product (control), KCREB, or the constitutively active CREB isoform CREB-DIEDML (24). In LacZ-expressing cells, MDI stimulated C/EBP β gene promoter activity by 3.5–4-fold (Fig. 6B). However, both basal and MDI-induced promoter activity was repressed in cells expressing KCREB. Alternately basal promoter activity was significantly elevated in cells expressing CREB-DIEDML, and further increases in transcription with MDI were not observed. These data suggest that CREB plays an important role in stimulating C/EBP β expression in response to MDI.

Dominant negative transcription factors may repress gene transcription by mechanisms apart from their ability to target specific trans-acting factors, including sequestration of co-activators and co-repressors serving as competitors for regulatory signaling enzymes, and transcriptional squelching (25–28). Therefore, to better define the role of CREB in C/EBP β gene expression, we used siRNA to deplete CREB and ATF1. Fig. 1A shows that this treatment did not reduce basal or MDI-stimulated levels of C/EBP β. This led us to hypothesize that perhaps other CRE-binding factors might stimulate C/EBP β expression in the absence of CREB/ATF1. Lin et al. (29) have reported that ATF2 and c-Jun increase C/EBP β gene transcription in vitro. The ability of CREM to bind CREs makes it another possible candidate for the regulation of C/EBP β expression.

To assess this hypothesis, C/EBP β gene promoter activity and C/EBP β expression were measured in untreated and MDI-treated 3T3-L1 cells transfected with siRNA to CREB/ATF1, ATF2, c-Jun, and CREM. Transcription from the C/EBP β gene promoter was stimulated by MDI to approximately the same level in 3T3-L1 cells transfected with individual siRNAs to each factor (Fig. 6C). On the other hand, basal and MDI-stimulated promoter activity was diminished when the siRNAs were combined. A similar response was noted in experiments in which C/EBP β protein levels were measured. In these studies, transfection of individual siRNAs for CREB/ATF1, ATF2, c-Jun, or

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antisense to CREM had no effect on the ability of MDI to induce C/EBP β expression (Fig. 6D). Likewise when siRNAs for ATF2, c-Jun, or CREM were transfected together with siRNA for CREB/ATF1, no change in the induction of C/EBP β by MDI was observed. Only when the siRNAs for all the factors were used in combination was MDI-induced C/EBP β expression attenuated. These data suggest that multiple CRE-binding factors may participate in the regulation of C/EBP β during adipogenic conversion.

Finally we examined the binding of CREB, ATF2, c-Jun, and CREM to an oligonucleotide probe comprising the distal CRE region (−117 to −96) in the C/EBP β gene promoter. Electrophoretic mobility shift assays were performed in the absence or presence of antibodies that recognize CREB, ATF2, c-Jun, and CREM. In the absence of nuclear extract protein from MDI-treated 3T3-L1 cells, no shifted band was observed (Fig. 6E). A single shifted band was present in reactions performed with nuclear extract and a nonspecific IgG as control. However, each of the reactions incubated with individual antibodies to CREB, ATF2, c-Jun, and CREM showed two shifted bands, one corresponding to the single band present in the IgG reactions and one corresponding to a supershifted band. The data indicate that each of the four CRE-binding factors can bind to the distal C/EBP β gene promoter CRE in MDI-treated cells.

**CREB Binds to the PPARγ2 Gene Promoter and Stimulates PPARγ2 Gene Transcription**—Fig. 1 shows that depletion of CREB/ATF1 prevented the induction of PPARγ2 expression by MDI although C/EBP β expression is stimulated under these conditions. This suggests that CREB/ATF1 may play a role in PPARγ2 gene expression. To test this concept, we assessed the ability of CREB-DIEDML to stimulate transcription from and bind to the PPARγ2 gene promoter. Treatment of confluent 3T3-L1 cells with MDI increased transcription from the PPARγ2 gene promoter by almost 7-fold within 48 h (Fig. 7A). Co-transfection of cells with a CREB-DIEDML expression vector stimulated PPARγ2 gene promoter activity by ∼4-fold, whereas ectopic expression of C/EBP β produced a modest 2-fold increase in promoter activity. When CREB-DIEDML and C/EBP β were co-expressed, PPARγ2 gene promoter activity was increased by 5–6-fold. Thus, activation of CREB (and ATF1) is sufficient to induce significant expression of PPARγ2 although to a lesser extent than MDI.

Inspection of the mouse PPARγ2 gene promoter indicates the presence of at least one potential CRE sequence (−143 to −137) (30). Chromatin immunoprecipitation assays were used to evaluate the binding of CREB to the PPARγ2 gene promoter. A PCR product corresponding to a 600-base-long region of the PPARγ2 gene promoter was detected among chromatin fragments immunoprecipitated with a CREB-specific antibody (Fig. 7B). No band was detected in material retrieved with nonspecific IgG. Neither antibody pulled down fragments of the glyceraldehyde-3-phosphate dehydrogenase gene lacking CRE sites. The results indicate that CREB binds the PPARγ2 gene promoter.

**DISCUSSION**

Depletion of CREB/ATF1 Blocks Adipogenesis in Vitro and in Vivo—In previous studies, we demonstrated that activation of CREB was necessary and sufficient to drive adipogenic conversion of 3T3-L1 cells in culture using constitutively active and dominant negative forms of CREB (16). Because the use of constitutively active and dominant negative factors can be plagued by nonspecific effects and interactions, we sought in the current experiments to confirm the importance of CREB in adipogenesis by specific knockdown with siRNA. Our CREB siRNA also decreased ATF1 levels but did not alter expression of ATF2, c-Jun, or CREM. Although we did not exhaustively investigate the impact of CREB siRNA on other CRE-binding proteins, we surmise that its lack of effect on the closely related factor CREM indicates that it does not affect the expression of other CRE-binding factors.

The results show that loss of CREB/ATF1 effectively blocks MDI-induced adipogenic conversion of 3T3-L1 cells in culture, confirming our previous observations. In addition, depletion of CREB/ATF1 completely blocked adipogenesis of 3T3-F442A cells implanted into athymic mice. Thus, the importance of CREB in adipocyte development is not merely a phenomenon of cultured cells but extends to in vivo conditions. These studies provide the rationale and motivation for ongoing studies to investigate the role of CREB in adipose tissue development and function in transgenic and knock-out animal models.

Depletion of Other CRE-binding Factors Does Not Affect Adipogenesis—Other investigators (21, 22) have reported that the expression and/or activation of ATF2 and c-Jun is increased by treatment of 3T3-L1 cells with differentiation-inducing agents. Both of the factors have been shown to stimulate C/EBP β gene transcription (29) and have been proposed to play central roles in the initiation of adipogenic conversion. Members of the CREM family of transcription factors, which are structurally related to CREB, are expressed in preadipocytes, although their participation in adipogenesis has not been explored. Our results indicate that the individual loss of any of these factors has little if any impact on adipocyte differentiation. Thus, despite their regulation by MDI, ability to bind CREs, and potential structural similarities to CREB, only CREB/ATF1 are essential for adipocyte development. These results provide further impe-
Depletion of CREB/ATF1 Blocks Adipogenesis Induced by Ectopic Expression of C/EBPs α and β or PPAR-γ2—In previous experiments, we demonstrated that expression of constitutively active forms of CREB in 3T3-L1 cells overcomes the inhibition of adipogenesis due to blockade of Ras/Raf/ERK signaling (17). These data highlighted CREB as a primary target for agents and signaling pathways that stimulate adipogenesis and as a crucial

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Depletion of CREB/ATF1 Blocks Adipogenesis Induced by Ectopic Expression of C/EBPs α and β or PPAR-γ2—In previous experiments, we demonstrated that expression of constitutively active forms of CREB in 3T3-L1 cells overcomes the inhibition of adipogenesis due to blockade of Ras/Raf/ERK signaling (17). These data highlighted CREB as a primary target for agents and signaling pathways that stimulate adipogenesis and as a crucial
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First CREB is not solely responsible for C/EBP β expression. Trautwein and co-workers (20) previously demonstrated the importance of the two CRE sites in the C/EBP β gene promoter to C/EBP β expression. Likewise we reported that inhibition of CREB via expression of dominant negative CREB isoforms repressed C/EBP β gene promoter activity and showed by chromatin immunoprecipitation that CREB binds the C/EBP β gene promoter (19). However, our current studies demonstrated that specific knockdown of CREB/ATF1 had no effect on C/EBP β expression. Likewise the individual knockdown of other CRE-binding factors like ATF2 and c-Jun, which have been implicated in C/EBP β gene expression (29), did not alter MDI-induced C/EBP β levels. Only the combined loss of CREB/ATF1, ATF2, c-Jun, and CREM was sufficient to repress MDI stimulation of C/EBP β expression and C/EBP β gene promoter activity. These results suggest that multiple CRE-binding factors participate in C/EBP β expression in response to MDI, perhaps in proportion to their relative abundance or activation state, and highlight the importance of C/EBP β in early adipogenic conversion.

Why then do dominant negative CREB isoforms repress C/EBP β expression, whereas depletion of CREB/ATF1 with siRNA does not? This probably reflects the ability of overexpressed, dominant negative factors to inhibit transcription via nonspecific interactions with other factors or by sequestration of co-adapters and other components of the transcriptional machinery. More detailed analysis of the transcription factors, co-activators, and co-repressors that bind the C/EBP β gene promoter is underway to better understand the complex regulation of this gene.

Second and perhaps more importantly, CREB/ATF1 appears to directly regulate PPARγ2 expression. This conclusion is based on the ability of CREB siRNA to block MDI-induced PPARγ2 expression although C/EBP β expression is unaffected. In addition, constitutively active CREB induces transcription from the PPARγ2 gene promoter to a greater extent than C/EBP β, and CREB binds to the promoter of the PPARγ2 gene as determined by chromatin immunoprecipitation. These results indicate that CREB not only participates in the early stages of adipogenesis but may also contribute to the persistent expression of PPARγ2 and other proadipogenic factors at later stages of the differentiation program. These observations also suggest that CREB may have a more central role in promoting PPARγ2 expression and adipogenesis than C/EBP β. In vitro, ectopic expression of C/EBP β promotes adipogenesis alone and enhances the adipogenesis in cells treated with MDI. Decreased C/EBP β activity attenuates adipogenic conversion of cultured preadipocytes. However, mice in which C/EBP β is knocked out exhibit diminished adiposity compared with wild type animals but still make adipose tissue (31). Thus, C/EBP β may not be essential to adipocyte development. The relative contribution of CREB and C/EBP β to adipose biology is the focus of future studies in our laboratory.
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**Figure 8. Model of CREB participation in adipogenesis.** Treatment of preadipocytes with cAMP mimetics and insulin stimulates CREB activation. Activated CREB, in combination with ATF2, CREM, and c-Jun, promotes the expression of C/EBP β. CREB and C/EBP β stimulate expression of PPARγ. PPARγ and C/EBP β drive expression of genes characteristic of the terminal adipocyte phenotype. CREB may also promote adipogenic conversion by increasing expression of Akt, cyclin D1, and PPARγ ligands and by repressing the expression of antiadipogenic factors like necdin and Wnt10a and Wnt10b.

**Model of CREB Activity in Adipogenesis**—How does CREB contribute to adipogenesis? Our previous and current studies suggest the model shown in Fig. 8. In this model, elevated intracellular cAMP levels (or treatment with cAMP mimetics) activate CREB and ATF1 via phosphorylation by protein kinase A (15). In addition, insulin and insulin-like growth factor-1 activate CREB through the ERK signaling pathway early in adipogenesis and through phosphatidylinositol 3-kinase/Akt during later stages. CREB may then drive expression of C/EBP β alone as postulated previously (19) or in combination with ATF2, c-Jun, or other factors as our current data suggest. In addition, CREB may directly induce expression of PPARγ, although the reason for the lag between CREB activation and delayed PPARγ expression is not clear. Interestingly, Farmer (9) has reported that CAMP-dependent signaling may stimulate PPARγ activity by increasing the expression of endogenous PPARγ ligands. Thus, CREB may promote PPARγ expression and activity via multiple mechanisms.

Limited gene array experiments revealed that activation of CREB promotes the expression of cyclin D1 and Akt-1 (18). Cyclin D1 expression is rapidly increased following MDI treatment and is required for mitotic clonal expansion prior to initiation of the differentiation program. siRNA-mediated depletion of cyclin D1 blocks MDI-stimulated adipogenic conversion of 3T3-L1 cells. Inhibition of phosphatidylinositol 3-kinase/Akt pathways in 3T3-L1 cells prevents adipogenesis (32), indicating the importance of this pathway to adipocyte development. In the absence of CREB, Akt levels may be insufficient to support robust adipocyte differentiation.

More extensive gene array studies were performed by Tseng et al. (33) to assess the role of insulin and insulin-like growth factor signaling targets in brown adipocyte differentiation. Their data revealed that expression of necdin and Wnt10a, two antiadipogenic factors that are highly expressed in preadipocytes, was repressed via insulin receptor substrate signaling. RNA interference-mediated knockdown of necdin restored the capacity to undergo adipocyte differentiation to cells lacking insulin receptor substrates. Additional studies indicated that CREB phosphorylation and activity were elevated by insulin receptor substrate signaling and that ectopic expression of a constitutively active CREB diminished necdin and Wnt10a levels. Thus, necdin and Wnt10a are also key targets for CREB during adipogenesis.

MacDougald and co-workers (34, 35) have demonstrated that Wnt10b also represses adipogenic conversion of 3T3-L1 and 3T3-F442A cells. They have shown that cAMP mimetics, but not insulin or dexamethasone, lead to decreased Wnt10b expression (4). Because CREB is the primary transcription factor target for cAMP/protein kinase A signaling, their data implicated CREB in the regulation of Wnt10b. We have found that ectopic expression of constitutively active CREB or depletion of CREB with siRNA reproducibly and predictably modulates transcription from the Wnt10 gene promoter.3

In conclusion, depletion of CREB and ATF1 attenuates adipogenesis of 3T3-L1 cells in culture or 3T3-F442A cells in vivo. Loss of other CRE-binding factors did not have the same effect. CREB/ATF1 depletion blocked adipogenesis of 3T3-L1 cells individually expressing the master adipogenic regulators C/EBP α or β or PPARγ2. Loss of CREB/ATF1 or other CRE-binding factors individually did not repress C/EBP β expression, whereas combined knockdown of these factors effectively inhibited MDI-induced C/EBP β expression. CREB binds and transactivates the PPARγ2 gene promoter. These results highlight the importance of CREB in the formation of new adipocytes and indicate that CREB participates in adipogenesis at various stages of the differentiation program.

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