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C/EBP transcription factors regulate *SREBP1c* gene expression during adipogenesis

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Abstract

The transcription factor SREBP1c (sterol-regulatory-element-binding protein 1c) is highly expressed in adipose tissue and plays a central role in several aspects of adipocyte development including the induction of PPAR γ (peroxisome-proliferator-activated receptor γ), the generation of an endogenous PPAR γ ligand and the expression of several genes critical for lipid biosynthesis. Despite its significance, the regulation of SREBP1c expression during adipogenesis is not well characterized. We have noted that in several models of adipogenesis, SREBP1c expression closely mimics that of known C/EBP β (CCAAT/enhancer-binding protein β) targets. Inhibition of C/EBP activity during adipogenesis by expressing either the dominant-negative C/EBP β LIP (liver-enriched inhibitory protein) isoform, the co-repressor ETO (eight-twenty one/MTG8) or using siRNAs (small interfering RNAs) targeting either C/EBP β or C/EBP δ significantly impaired early SREBP1c induction. Furthermore, ChIP (chromatin immunoprecipitation) assays identified specific sequences in the SREBP1c promoter to which C/EBP β and C/EBP δ bind in intact cells, demonstrating that these factors may directly regulate SREBP1c expression. Using cells in which C/EBP α expression is inhibited using shRNA (short hairpin RNA) and ChIP assays we show that C/EBP α replaces C/EBP β and C/EBP δ as a regulator of SREBP1c expression in maturing adipocytes. These results provide novel insight into the induction of SREBP1c expression during adipogenesis. Moreover, the findings of the present study identify an important additional mechanism via which the C/EBP transcription factors may control a network of gene expression regulating adipogenesis, lipogenesis and insulin sensitivity.

Keywords

adipocyte; adipogenesis; CCAAT/enhancer-binding protein (C/EBP); lipid; sterol-regulatory-element-binding protein 1c (SREBP1c); transcription

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AUTHOR CONTRIBUTION

Victoria Payne and Wo-Shing Au performed the majority of the experimental work, and Christopher Lowe performed some minor additional experiments. Shaikh Rahman and Jacob Friedman provided samples from *Cebpb*-knockout mice. Stephen O'Rahilly provided additional advice, while Justin Rochford initiated the study, designed and supervised the experiments and drafted the manuscript. All authors contributed to the discussion of data, proposed improvements to the experimental work and revision of the manuscript.

INTRODUCTION

Adipose tissue is a complex, highly active metabolic and endocrine organ [1]. While the adverse metabolic consequences of excessive adiposity are well known, pathologically decreased lipid accumulation or impaired adipogenesis in lipodystrophic subjects also has similar deleterious metabolic consequences including insulin resistance, dyslipidaemia and the associated risk of cardiovascular disease [2,3]. Thus optimal metabolic health probably requires the restraint of adipose tissue mass while still maintaining the capacity to respond accurately to substrate availability to increase adipose mass when required. A fuller understanding of the pathways regulating the formation and maintenance of adipocytes is therefore likely to inform therapeutic strategies for the treatment of syndromes involving either decreased or increased adiposity.

The development of new adipocytes from pluripotent precursors involves a complex and tightly orchestrated programme of gene expression [1]. Key early regulators of this process are C/EBP β and C/EBP δ (CCAAT/enhancer-binding proteins β and δ). Acting alongside multiple co-activators, co-repressors and other transcription factors they play a central role in the subsequent induction of PPAR γ (peroxisome-proliferator-activated receptor γ) and C/EBP α , transcription factors that have been dubbed the ‘master regulators of adipogenesis’ owing to their critical role in this process. The targets of these transcription factors include the promoters of many genes of the mature lipogenic and insulin-sensitive adipocyte such as aP2 (adipocyte protein 2), PEPCK (phosphoenolpyruvate carboxykinase), lipoprotein lipase, adiponectin and GLUT4 (glucose transporter 4) [4–6]. Thus the C/EBP family of transcription factors has a critical role in adipocyte development and lipid accumulation. Studies investigating the importance of C/EBP β and C/EBP δ have demonstrated that loss of one or both of these factors can lead to decreased adipose mass in mice and decreased adipogenesis in cellular models [7,8]. In addition C/EBP factors may directly influence lipogenesis by controlling the early induction of the key lipogenic enzyme DGAT2 (diacylglycerol acyltransferase 2) during adipogenesis [9].

SREBP1c (sterol-regulatory-element-binding protein 1c) is another important pro-adipogenic transcription factor that can directly regulate the expression of several key genes of lipid metabolism [10]. Moreover, in adipocyte differentiation SREBP1c appears to contribute both to the expression of PPAR γ and the production of an endogenous PPAR γ ligand [11,12]. SREBP1c expression and activity, via cleavage and nuclear translocation, are acutely responsive to insulin [10,13]. In addition to controlling genes involved in lipid metabolism, the regulation of the expression of the adipokines leptin and adiponectin by insulin is also mediated by SREBP1c [14,15]. Thus in both developing and mature adipocytes, SREBP1c can potentially integrate information of nutritional and metabolic status to control new adipocyte formation, lipid metabolism, insulin sensitivity and, via adipokines, whole-body energy homeostasis and appetite. Despite the importance of SREBP1c and its established role in adipocyte development, relatively little is known about the factors controlling its expression during adipogenesis, although LXR α (liver X receptor α) has been shown to be important for SREBP1c expression in these cells [16]. In the present study, we demonstrate that the C/EBP family of transcription factors also play a critical role in both the early induction of SREBP1c and the maintenance of its expression in maturing adipocytes.

EXPERIMENTAL

Preadipocyte isolation and cell culture

Human preadipocytes were grown from the stromovascular fraction of collagenase-digested abdominal subcutaneous adipose tissue as previously described [17]. At various times

following induction of differentiation, cells were harvested, and RNA was extracted. 3T3-L1 preadipocytes were maintained and differentiated as described in [18]. 3T3-L1 preadipocyte cells stably expressing the LIP (liver-enriched inhibitory protein) isoform of C/EBP β or ETO (eight-twenty one/MTG8) were as described previously [9]. Differentiating 3T3-L1 cells were assessed for lipid content by staining with Oil Red O as described in [18]. Murine E14 (where E is embryonic day) ES (embryonic stem) cells were cultured and differentiated as described in [19].

siRNA (small interference RNA) knockdown

Synthetic double-stranded siRNAs against C/EBP β or C/EBP δ mRNAs were purchased from Ambion. 3T3-L1 preadipocytes were plated at a density 1×10^5 cells per well in 12-well plates the day before siRNA transfection. siRNA/liposome mixes containing 2 μ g of LipofectamineTM 2000 (Invitrogen) and 100 nM siRNA/well were incubated with cells for 6 h in the absence of serum. Medium was replaced with serum containing 3T3-L1 growth medium for 18 h prior to the induction of differentiation. 3T3-L1 preadipocytes stably expressing shRNA (short hairpin RNA) sequences targeting C/EBP α were generated using the pSiren retroQ kit (BD Biosciences) according to the manufacturer's protocol. Retrovirus production, 3T3-L1 infection and selection were essentially as described in [18].

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was extracted from cell cultures using an RNeasy kit (Qiagen). Adipose tissue was isolated from *Cebpb*-null mice or their wild-type littermates and RNA was isolated as previously described [20]. All procedures were approved by the UCHSC Animal Care and Use Committee. Primer Express, version 1.0 software (PerkinElmer Applied Biosystems), was used to design the probes and primers for real-time quantitative PCR to determine *Srebp1c*, *Srebp1a*, *Dgat2*, *Pparg2*, *aP2*, *Glut4*, *Cebpb* and *Cebpd* mRNA expression. A primer/probe mix to assay *Cebpa* was obtained from Applied Biosystems. RNA was reverse-transcribed using Moloney-murine-leukaemia virus reverse transcriptase and random hexamer primers (Promega). The resulting cDNA was used in 12 μ l PCR mixtures, in which 300 nmol/l forward and reverse primers and, where applicable, 150 nmol/l fluorogenic probe were used in combination with ABI Taqman or SYBR[®] Green Master Mix (Applied Biosystems). Reactions were carried out in duplicate for each sample on an ABI 7900 sequence detection system (PerkinElmer Biosystems) according to the manufacturer's instructions. The relative quantities of amplified cDNAs were analysed with the SDS software (Applied Biosystems) and target values were normalized to 18S rRNA (tissue samples) or cyclophilin A mRNA (cell culture samples).

ChIP (chromatin immunoprecipitation) assay

3T3-L1 preadipocytes in 35-mm wells were differentiated for various times as indicated. The DNA and protein were cross-linked *in situ* with 0.5 % formaldehyde at 37 °C for 5 min. Soluble chromatin was prepared using a ChIP assay kit (Upstate Biotechnology). The lysate was sonicated four times for 10 s at 4 °C. The lysates were precipitated with either 5 μ l of antiC/EBP β , anti-C/EBP δ or anti-C/EBP α antibody (Santa Cruz Biotechnology) overnight before Protein A-agarose beads were added. DNA was recovered by digesting with 10 μ g/ml proteinase K at 45 °C for 30 min and purification using a QIAquick PCR purification kit (Qiagen). The presence of DNA sequences associated with the immunoprecipitated proteins was determined using specific primers amplifying DNA sequences, including the binding sites being assayed, and SYBR[®] Green Master Mix according to the manufacturer's protocol. Values obtained from immunoprecipitated samples were normalized to those from input samples.

Western blot analysis

Protein samples were extracted by scraping in lysis buffer [50 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM Na₃VO₄, 30 mM NaF, 10 mM Na₄P₂O₇, 2.5 mM benzamidine, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 1 µg/ml antipain, 0.5 mM PMSF and 1 % Triton X-100] containing 1 % Nonidet P40, followed by sonication as described previously [18]. After centrifugation for 10 min at 13 000 g, samples of supernatant containing 30 µg of protein were denatured and analysed by Western blotting. All antibodies were from Santa Cruz Biotechnology.

Statistics

Statistical analyses were performed using Student's *t* test or ANOVA for multiple comparisons.

RESULTS

The gene *Srebp1l* encodes the proteins SREBP1a and SREBP1c which have highly overlapping specificities for target sequences, but appear to differ in their transactivation capacities [21]. While both SREBP1a and SREBP1c are expressed in adipose tissue *in vivo*, SREBP1c is the more highly expressed in this tissue in both mice and humans [22]. However, initial studies suggested that only the SREBP1a isoform was expressed in differentiating 3T3-L1 cells [22], although other investigators have reported substantial expression of SREBP1c [12]. We therefore examined the expression of each isoform in differentiating 3T3-L1 preadipocytes. As shown in Figure 1(A), *SREBP1c* mRNA expression was almost undetectable in preadipocytes, but dramatically induced during adipogenesis and abundant in 3T3-L1 adipocytes. In contrast *SREBP1a* was clearly detectable in preadipocytes and, while also induced further during differentiation, did not show the dramatic increases observed for *SREBP1c* (Figure 1B). These assays do not permit quantitative comparison of *SREBP1a* versus *SREBP1c* levels, however, they do show that *SREBP1c* is highly induced and abundant during adipogenesis, making 3T3-L1 cells suitable to study the transcriptional regulation of *SREBP1c*. When searching for candidate pathways that might regulate SREBP1c we noted that the time course of its induction was similar to that observed for known C/EBPβ targets such as C/EBPα, 11βHSD1 (11-β-hydroxysteroid dehydrogenase type 1) and DGAT2 [9,23,24]. In addition we found that SREBP1c and the C/EBPβ target DGAT2 were induced with similar time courses in both murine ES cells undergoing adipogenic differentiation (Supplementary Figures S1A and S1B at <http://www.BiochemJ.org/bj/425/bj4250215add.htm>) and differentiating isolated human preadipocytes (Supplementary Figures S1C and S1D).

To further investigate whether C/EBPβ might regulate *SREBP1c* expression we examined its expression in cells over-expressing the inhibitory LIP isoform of C/EBPβ and cells constitutively expressing the C/EBPβ inhibitor ETO [9]. As shown in Figures 1(C) and 1(D) respectively, LIP expression inhibited not only C/EBPα expression but also the induction of *SREBP1c* mRNA. Likewise, inhibition of C/EBP activity with ETO impaired the induction of C/EBPα (Figure 1E) and *SREBP1c* expression (Figure 1F) to a similar degree. Taken together these results support a role for C/EBPβ in the regulation of SREBP1c during adipogenesis.

LIP is known to inhibit multiple C/EBP isoforms, whereas the specificity of ETO is not fully defined. Therefore we next used specific siRNA oligonucleotides to knockdown individual C/EBP isoforms to examine their contribution to the regulation of SREBP1c. Cells were transfected with siRNAs targeting C/EBPβ or C/EBPδ or a mixture of both prior to induction of differentiation. As shown in Figure 2(A), the increase in C/EBPβ expression

following the induction of differentiation was inhibited by transfection with *C/EBPβ* siRNA, but not *C/EBPδ* siRNA. A similar degree of inhibition was observed when *C/EBPβ* siRNA was co-transfected with *C/EBPδ* siRNA. Likewise *C/EBPδ* siRNA significantly impaired the induction of *C/EBPδ* expression, but did not prevent *C/EBPβ* induction (Figure 2B). *C/EBPδ* siRNA was similarly effective when co-transfected with *C/EBPβ* siRNA, substantially inhibiting *C/EBPδ* expression.

Lysates from identically transfected cells differentiated for various times were analysed by Western blotting to determine either *C/EBPβ* or *C/EBPδ* protein expression (Figure 2C). Knockdown of *C/EBPβ* or *C/EBPδ* with siRNA resulted in an almost complete loss of the induction of *C/EBPβ* (top panel) or *C/EBPδ* (bottom panel) protein respectively, consistent with the effects observed at the RNA level. Again, co-transfection of cells with siRNA targeting both *C/EBPβ* and *C/EBPδ* led to almost complete inhibition of the expression of both of these proteins. As we have previously reported [9], knockdown of *C/EBPβ* led to an almost complete inhibition of lipid accumulation in differentiating 3T3-L1 cells as assessed by Oil Red O staining (Figure 2D). Knockdown of *C/EBPδ* expression had a less marked effect on lipid accumulation in these cells. The knockdown of both *C/EBPβ* and *C/EBPδ* together led to the most dramatic inhibition of lipid accumulation.

We next examined the effect of *C/EBPβ* and *C/EBPδ* knockdown on a well-characterized *C/EBP* target gene, *C/EBPα*. As predicted by previous cellular and *in vivo* studies, *C/EBPα* expression was most strongly affected by inhibition of *C/EBPβ*, although *C/EBPδ* knockdown also substantially inhibited its induction during adipogenesis (Figure 2E). Knockdown of both *C/EBPβ* and *C/EBPδ* in the same cells further impaired the induction of *C/EBPα* mRNA. Having shown that *C/EBPβ* regulates *DGAT2* induction during adipogenesis in a previous study [9], we also examined whether *C/EBPδ* could regulate *DGAT2* expression. Indeed, knockdown of *C/EBPδ* significantly inhibited *DGAT2*, which was reduced to 45 % and 50 % of the levels seen in control cells at day 3 and day 5 of differentiation respectively (Figure 2F). In addition, the combined knockdown of both *C/EBPβ* and *C/EBPδ* caused an even greater reduction in *DGAT2* expression. These results suggest that, in addition to *C/EBPβ*, *C/EBPδ* makes an important contribution to the regulation of *DGAT2* expression during early adipogenesis.

Analysis of *SREBP1c* expression in these samples revealed that the regulation of *SREBP1c* was very similar to that of *C/EBPα* and *DGAT2*. Knockdown of *C/EBPβ* alone led to inhibition of *SREBP1c* expression to approx. 30 % and 50 % of the levels seen in control cells at day 3 and day 5 respectively, following differentiation of these cells (Figure 2G). Inhibition of *C/EBPδ* using siRNA gave very similar results, suggesting that both *C/EBPβ* and *C/EBPδ* are important for the induction of *SREBP1c* during adipogenesis. Moreover, siRNA knockdown of both *C/EBPβ* and *C/EBPδ* in the same cells reduced expression of *SREBP1c* at day 3 and day 5 further to approx. 20 % and 35 % of those in control cells respectively.

To determine the specificity of these effects, the effect of *C/EBPβ* and *C/EBPδ* inhibition on *SREBP1a* expression was also determined. As shown in Figure 2(H), the inhibition of *C/EBPβ*, *C/EBPδ* or combined inhibition of both factors by siRNA did not significantly affect *SREBP1a* expression at any of the time points tested. These results show that, as *SREBP1a* is normally induced in these cells, *C/EBPβ/δ* knockdown does not inhibit all changes associated with adipogenesis, but rather is specific to genes downstream of these factors. These data also indicate that the two isoforms of *SREBP* are regulated by different factors during adipogenesis.

To determine whether our observations in 3T3-L1 cells may extend to *in vivo* adipocyte development, we examined gene expression in white adipose tissue isolated from *Cebpb*-knockout mice. *Cebpb* mRNA was undetectable in these samples (Figure 3A), while the expression of *Cebpa* was significantly reduced (Figure 3B), as previously reported [9]. Consistent with our results from cultured cells, *Srebp1c* expression was significantly decreased in the white adipose tissue of these mice (Figure 3C). This suggests that C/EBP β is also involved in the expression of SREBP1c in adipocytes *in vivo*. In addition, the expression of *Srebp1a* was not affected in mice lacking *Cebpb* (Figure 3D), providing *in vivo* support for our previous findings in cultured preadipocytes that SREBP1c, but not SREBP1a, is selectively regulated by C/EBP factors. We next sought to determine whether C/EBP β and C/EBP δ could directly regulate SREBP1c through binding to its promoter. Examination of the putative promoter of SREBP1c revealed several potential C/EBP consensus-binding sites within the 4 kb upstream of the transcriptional start site. To assess binding to these putative sites we performed ChIP analysis, immunoprecipitating C/EBP β or C/EBP δ , and using real-time PCR to quantify binding to specific DNA sequences. Of nine putative binding sites identified, three showed significant binding of either C/EBP β or C/EBP δ , which was responsive to the induction of adipogenesis, whereas the other six did not (results not shown). The three responsive sites were designated site 1, site 2 and site 3, and are schematically represented in Figure 4(A). As shown in Figures 4(B)–4(D), C/EBP β binding to all three sites was increased during early differentiation, as C/EBP β expression increases. Similarly these sites also bound C/EBP δ with similar time courses in identically treated cells (Figures 4E–4G). Taken together, these results strongly suggest that both C/EBP β and C/EBP δ are direct upstream regulators of SREBP1c expression, directly binding to the SREBP1c promoter during early adipogenesis.

As has been previously described for the C/EBP α and DGAT2 promoters, among others, the maximal binding of C/EBP β and C/EBP δ to the SREBP1c promoter lags behind the induction of these factors by several hours, probably due to the binding of inhibitory factors such as CHOP10 (C/EBP-homologous protein 10) and ETO. Similarly, the maximal binding of C/EBP β and C/EBP δ precedes the peak of SREBP1c expression as has previously been observed for other well-characterized C/EBP β / δ targets, probably due to the formation of inactive promoter-bound complexes that must be de-repressed for SREBP1c expression to occur.

We have previously observed that C/EBP α replaces C/EBP β as a major regulator of DGAT2 expression when the expression of the latter diminishes in the later stages of adipogenesis [9]. Thus we investigated whether C/EBP α might similarly take over the regulation of SREBP1c expression from C/EBP β and C/EBP δ as adipogenesis progresses. To test this we generated 3T3-L1 cells stably expressing shRNA targeting C/EBP α to inhibit its expression. This led to a significant inhibition of C/EBP α induction, which was reduced by 80 % or greater following induction of differentiation (Figure 5A). Consistent with the reciprocal regulation of PPAR γ and C/EBP α during adipogenesis, C/EBP α inhibition also led to significant reduction in the expression of PPAR γ in cells differentiated for 5 days (Figure 5B). The expression of key markers of the maturing adipocyte, including the fatty-acid-binding protein *aP2* and the insulin-sensitive *GLUT4*, were also suppressed in cells in which C/EBP α had been knocked down. Similarly, the expression of *DGAT2* was significantly inhibited in these cells, consistent with our previous study using preadipocytes transiently transfected with siRNA targeting C/EBP α [9]. In the same cells *SREBP1c* expression was found to be inhibited by approx. 70 % at day 5 of differentiation. In contrast, the loss of C/EBP α had no significant effect on the expression of the *SREBP1a* isoform.

To determine whether the regulation of SREBP1c by C/EBP α could occur through direct binding of the same sites in the SREBP1c promoter occupied in early adipogenesis by C/

EBP β and C/EBP δ , we performed ChIP assays. C/EBP α was immunoprecipitated from 3T3-L1 preadipocytes differentiated for various times and bound DNA sequences corresponding to putative C/EBP-binding sites were assayed by real-time PCR. This revealed that site 1 inducibly binds C/EBP α as adipogenesis proceeds, replacing the binding by C/EBP β and C/EBP δ 96 h after induction of differentiation (Figure 5C). In contrast, relatively weak binding was observed for C/EBP α to sites 2 and 3, and the marginal increases detected as adipogenesis progressed were not significant (Figures 5D and 5E).

Taken together, these results suggest that, during the later stages of adipogenesis, C/EBP α may substitute for C/EBP β and C/EBP δ in the control of SREBP1c expression and that this involves binding to site 1 approx. 3.5 kb upstream of the transcriptional start site in the SREBP1c promoter.

DISCUSSION

SREBP1c plays a central role in lipid metabolism, particularly in the liver and adipose tissue. While the regulation of SREBP1c in the liver has been extensively studied, the factors regulating the induction of SREBP1c in developing adipocytes has received less attention, despite the important role acknowledged for this protein in adipocyte development. Our results are the first to show a key direct role for C/EBP factors in the regulation of SREBP1c with C/EBP β and C/EBP δ initially binding to the SREBP1c promoter and subsequent regulation by C/EBP α . Selective siRNA- or shRNA-mediated knockdown demonstrated that the loss of any of these C/EBP factors significantly impairs the induction of SREBP1c. In addition, our results suggest that, at least for C/EBP β , this regulatory mechanism is likely to operate *in vivo*, as mice lacking C/EBP β also have reduced SREBP1c levels in adipose tissue.

While the present study places C/EBP β upstream of SREBP1c during adipogenesis, the C/EBP β promoter has conversely been described as a direct target of SREBP1c in mature adipocytes [25]. In this instance SREBP1c appears to be at least in part responsible for the induction of C/EBP β in response to insulin. This illustrates that, rather than acting in a fixed canonical cascade, these genes function in a complex inter-regulatory network, the order of which will depend on factors including extracellular signals and the differentiation status of the cells involved.

From the present study it is difficult to discriminate the relative importance of C/EBP β and C/EBP δ activity directly compared with the effects of consequent reduced expression of C/EBP α in the control of SREBP1c and lipogenesis. Forced expression of PPAR γ in fibroblasts from *Cebpa*-null mice permits appropriate induction of adipocyte genes such as aP2, GLUT4 and adiponectin during adipogenesis, but with significantly reduced lipid accumulation [26,27]. We have previously shown that DGAT2, a key enzyme of lipogenesis, is a direct target of C/EBP α in adipocytes [9]. We now show that the effect of DGAT2 loss in cells with impaired C/EBP function may be exacerbated by the decreased expression of SREBP1c and the panoply of lipogenic genes it regulates. The precise relative importance of direct C/EBP α -mediated compared with indirect SREBP1c-mediated pathways in the control of lipogenesis will require the specific knockdown of SREBP1c.

Given the complex interacting network of transcription factors involved in adipocyte differentiation, it is also difficult to determine the contribution of direct compared with indirect actions of C/EBP factors in regulating the SREBP1c promoter. C/EBPs have important roles in inducing the expression of many proadipogenic transcription factors, notably PPAR γ , and several of these are likely to regulate SREBP1c expression themselves. However, our ChIP assay data, clearly showing physical binding of C/EBPs to the SREBP1c

promoter in intact differentiating adipocytes, strongly suggests that the C/EBPs make a significant contribution to SREBP1c expression as primary regulators. Moreover, the observation that knockdown of C/EBP β expression does not affect C/EBP δ induction and vice versa strengthens the case that SREBP1c inhibition in each case does not result from an overall impairment of adipogenesis, but rather a selective inhibition of downstream genes.

It is interesting that C/EBP inhibition selectively inhibited SREBP1c, but not SREBP1a, expression. The relative importance of the SREBP1a and SREBP1c isoforms in different cultured models of adipogenesis has been controversial. Although specific inhibition of these two isoforms has not been compared in cultured preadipocytes, the results of the present study are consistent with a more important role for SREBP1c in adipogenesis. In the absence of C/EBP β , C/EBP δ and/or C/EBP α induction, lipogenesis, adipogenesis and SREBP1c expression are coordinately impaired, while SREBP1a expression is unaffected. Although this does not demonstrate that loss of SREBP1c alone would replicate this phenotype, it does suggest that, of the two isoforms, SREBP1c is more tightly linked to adipocyte development and lipogenesis. Given that both SREBP1a and SREBP1c appear to have near identical target specificities and that SREBP1a is the more potent, at least in cultured hepatocytes [21], it is not clear why this is the case. Cellular studies selectively inhibiting the SREBP1 isoforms during adipogenesis using shRNA will be valuable in dissecting their relative importance, particularly as attempts to understand specific SREBP1 function *in vivo* using animal models has given confusing results. Loss of both SREBP1 isoforms caused significant embryonic lethality and up-regulation of SREBP2 in surviving mice [28], while selective ablation of SREBP1c led to reduced hepatic expression of lipogenic gene expression but no overt adipose phenotype, although epididymal fat mass was reduced [29]. Paradoxically, adipose-specific overexpression of constitutively active nuclear SREBP1 in mice led to a complex syndrome of lipodystrophy [30]. Other important regulators of adipogenesis, such as PPAR γ and C/EBP α , have required more complex *in vivo* models, including chimaeric and hypomorphic mice and inducible postnatal knockouts to circumvent mortality *in utero* [31–33]. Similar models may be required to clarify the true importance of SREBP1c in adipose tissue development *in vivo*.

From a pathophysiological perspective, SREBP1c levels may be decreased in obese and Type 2 diabetic subjects [34,35], while altered SREBP1c levels have also been reported in lipodystrophic HIV patients undergoing antiretroviral therapy [36]. Furthermore, altered SREBP1c function may underlie or exacerbate lipodystrophy in patients with mutations in lamin A/C [37,38]. Thus a fuller understanding of how SREBP1c expression and/or activity may be modulated could be of significant therapeutic benefit in these conditions.

In summary, in the present study we have demonstrated for the first time that SREBP1c is regulated directly by C/EBP factors during adipocyte differentiation. This provides novel insight into the poorly defined transcriptional regulation of SREBP1c in developing adipose tissue, a key site of its action. Given the pleiotropic effects of SREBP1c, it identifies an additional mechanism via which C/EBP factors can indirectly influence lipid homeostasis and insulin action. Such further delineation of the complex network controlling adipocyte development is important for the development of therapeutic strategies to treat diseases featuring altered adipose tissue mass or function, including obesity and lipodystrophies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

aP2	adipocyte protein 2
C/EBP	CCAAT/enhancer-binding protein
ChIP	chromatin immunoprecipitation
DGAT2	diacylglycerol acyltransferase 2
ES cell	embryonic stem cell
ETO	eight-twenty one/MTG8
GLUT4	glucose transporter 4
LIP	liver-enriched inhibitory protein
PPAR	peroxisome-proliferator-activated receptor
shRNA	short hairpin RNA
siRNA	small interfering RNA
SREBP	sterol-regulatory-element-binding protein

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Figure 1. Inhibition of C/EBP β activity by constitutive LIP or ETO expression inhibits the induction of SREBP1c during adipogenesis

3T3-L1 preadipocytes were induced to differentiate for various times up to 8 days (D8), RNA was isolated and the expression of *SREBP1c* (A) or *SREBP1a* (B) was determined by real-time PCR. Results shown are normalized to cyclophilin A, and are means $\pm$ S.E.M. ($n = 4$). (C) 3T3-L1 preadipocytes were stably transfected with LIP (solid bars) or empty vector (open bars), and confluent cells were induced to differentiate for the times shown. RNA was isolated and the expression of *C/EBP α* mRNA determined by real-time PCR. (D) Identical samples were also assayed for *SREBP1c* mRNA. (E) *C/EBP α* mRNA expression was determined in differentiating 3T3-L1 preadipocytes stably transfected with empty vector (open bars) or ETO (solid bars). (F) *SREBP1c* mRNA expression was also determined in the same samples. All results shown are normalized to cyclophilin, and are means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$, compared with mock-transfected cells at the same time point. AU, arbitrary units.

Figure 2. siRNA knockdown of *C/EBP* β and/or *C/EBP* δ activity inhibits *SREBP1c* induction during adipogenesis

(A) 3T3-L1 preadipocytes were transfected with control siRNA (open bars) or siRNA targeting *C/EBP* β (light grey bars), *C/EBP* δ (dark grey bars) or a mixture of siRNA targeting both *C/EBP* β and *C/EBP* δ (solid bars), and were subsequently induced to differentiate for various times. *C/EBP* β mRNA expression was assayed by real-time PCR. (B) *C/EBP* δ mRNA expression was assayed in the same samples. In both cases values are means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$, compared with control siRNA transfected cells at the same time point. (C) Control, *C/EBP* β (siC β), *C/EBP* δ (siC δ) and *C/EBP* β +*C/EBP* δ (siC β + δ) siRNA-transfected cells were also assayed for *C/EBP* β [LIP/LAP (liver-enriched activating protein)] and *C/EBP* δ protein expression by Western blot analysis. (D) Lipid accumulation was assessed by Oil Red O staining in control, *C/EBP* β , *C/EBP* δ and *C/EBP* β +*C/EBP* δ siRNA-transfected cells following differentiation for 8 days. (E–H) Control (open bars), *C/EBP* β (light grey bars), *C/EBP* δ (dark grey bars) and *C/EBP* β +*C/EBP* δ (solid bars) siRNA-transfected cells were also differentiated for various times, RNA was isolated and assayed for the expression of *C/EBP* α (E), *DGAT2* (F), *SREBP1c* (G) and *SREBP1a* (H) by real-time PCR. Results shown are normalized to cyclophilin, and are means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$, compared with control siRNA-transfected cells at the same time point.

Figure 3. SREBP1c, but not SREBP1a, expression is reduced in adipose tissue of mice lacking C/EBP β

RNA was isolated from white adipose tissue of wild-type (WT) or *Cebpb*-knockout (KO) mice, reverse-transcribed and the expression of *Cebpb* (A), *Cebpa* (B), *Srebp1c* (C) and *Srebp1a* (D) was assayed by real-time PCR. Results are normalized to 18S rRNA, and are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$, compared with wild-type mice. AU, arbitrary units.

Figure 4. Identification of C/EBP β - and C/EBP δ -binding sites in the SREBP1c promoter
 (A) Analysis of the 4 kb upstream of the first exon of SREBP1c by TESS (transcription element search software) revealed three potential C/EBP β -binding sites, denoted site 1, site 2 and site 3. 3T3-L1 preadipocytes were induced to differentiate for various times and ChIP analysis performed to assess binding of C/EBP β to the putative site 1 (B), site 2 (C) and site 3 (D). C/EBP δ binding to the same sites was also determined (E–G). C/EBP β - and C/EBP δ -bound DNA in immunoprecipitates was quantified by real-time PCR. Values in each sample were normalized to total genomic DNA of the same sequence in the ‘input’ starting sample prior to immunoprecipitation. Values are means $\pm$ S.E.M. from four independent experiments. * P < 0.05, compared with binding at 0 h. AU, arbitrary units.

Figure 5. *C/EBPα* regulates *SREBP1c* expression in the later stages of adipogenesis
(A) 3T3-L1 preadipocytes stably expressing a control shRNA (open bars) or shRNA targeting *C/EBPα* (solid bars) were induced to differentiate for various times, RNA was extracted and the expression of *C/EBPα* mRNA was determined by real-time PCR. **(B)** Day 5 (D5) differentiated samples from cells expressing control (open bars) or *C/EBPα*-targeting shRNA (solid bars) were assayed for the expression of *PPARγ2* (*Pγ2*), *aP2*, *GLUT4* (*GLT4*), *DGAT2* (*DGT2*), *SREBP1c* (*SR1c*) and *SREBP1a* (*SR1a*). Values are means $\pm$ S.E.M., normalized to cyclophilin ($n = 3$). * $P < 0.05$, compared with control shRNA-transfected cells at the same time point. **(C–E)** 3T3-L1 preadipocytes were induced to differentiate for various times and subjected to ChIP analysis to assess binding of *C/EBPα* to the putative site 1 (**C**), site 2 (**D**) and site 3 (**E**) indicated in Figure 4(A). *C/EBPα*-bound DNA in immunoprecipitates was quantified by real-time PCR. Values in each sample were normalized to total genomic DNA of the same sequence in the ‘input’ starting sample prior to immunoprecipitation. Values are means $\pm$ S.E.M. from four independent experiments. * $P < 0.05$, compared with binding at 0 h. AU, arbitrary units.