Cyclic AMP Activates the Expression of Stearoyl-CoA Desaturase Gene 1 during Early Preadipocyte Differentiation

(Received for publication, May 2, 1996, and in revised form, August 26, 1996)

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Stearoyl-CoA desaturase gene 1 (scd1) mRNA levels were induced during the 1st day of 3T3-L1 differentiation. scd1 expression had previously been observed only in late-stage differentiation, during the period of triacylglycerol accumulation. The induction of 3T3-L1 differentiation requires treatment with insulin, dexamethasone, and an agent that increases intracellular cAMP levels. Treatment of preadipocytes with cAMP-elevating agents caused an increase in scd1 mRNA concentrations. Insulin and dexamethasone had no effect on scd1 mRNA expression in preadipocytes. The increase in mRNA resulted from transcriptional activation of the scd1 gene. 8-Bromo-cAMP treatment of differentiated adipocytes had no effect on scd1 mRNA levels, suggesting a preadipocyte-specific effect. The increase in scd1 mRNA occurred maximally after 6 h of cAMP treatment and was shown to require protein synthesis. Deletion and mutagenesis analyses have localized the cAMP response to sequence within the first 250 base pairs of the 5′-flanking region of the scd1 gene. Treatment with phorbol ester enhanced the induction of scd1 mRNA by cAMP, suggesting the involvement of AP-2 as a mediator of the cAMP response. The induction of scd1 expression by cAMP during early differentiation was distinct from that observed during late adipocyte development. This early expression presents a novel regulated function of scd1 during the differentiation process, independent of its lipogenic role in late adipocyte development. The induction of scd1 also introduces a role for cAMP in 3T3-L1 differentiation.

The 3T3-L1 preadipocyte cell line, originally obtained from mouse embryos (1), provides a reliable model for characterizing the events responsible for adipocyte differentiation (2). In culture, the cells are induced to differentiate upon treatment with methylisobutylxanthine (a cyclic-AMP phosphodiesterase inhibitor), dexamethasone (a synthetic glucocorticoid agonist), and an agent that increases intracellular cAMP levels. Treatment of preadipocytes with cAMP-elevating agents caused an increase in scd1 mRNA concentrations. Insulin and dexamethasone had no effect on scd1 mRNA expression in preadipocytes. The increase in mRNA resulted from transcriptional activation of the scd1 gene. 8-Bromo-cAMP treatment of differentiated adipocytes had no effect on scd1 mRNA levels, suggesting a preadipocyte-specific effect. The increase in scd1 mRNA occurred maximally after 6 h of cAMP treatment and was shown to require protein synthesis. Deletion and mutagenesis analyses have localized the cAMP response to sequence within the first 250 base pairs of the 5′-flanking region of the scd1 gene. Treatment with phorbol ester enhanced the induction of scd1 mRNA by cAMP, suggesting the involvement of AP-2 as a mediator of the cAMP response. The induction of scd1 expression by cAMP during early differentiation was distinct from that observed during late adipocyte development. This early expression presents a novel regulated function of scd1 during the differentiation process, independent of its lipogenic role in late adipocyte development. The induction of scd1 also introduces a role for cAMP in 3T3-L1 differentiation.

Late marker genes consist of lipogenic and lipolytic genes, as well as other genes responsible for modulating the mature adipocyte phenotype. During days 3–7 of the differentiation program, fat droplets appear and increase in size as the cells acquire the morphological and biochemical characteristics of terminally differentiated adipocytes.

One of the lipogenic genes known to be expressed during late differentiation is scd1. scd1 encodes for an isozyme of stearoyl-CoA desaturase, a key enzyme involved in the biosynthesis of unsaturated fatty acids and the regulation of fatty acid biosynthesis. scd1 catalyzes the 9-cis desaturation of fatty acyl-CoAs, the major products being palmitoleoyl- and oleoyl-CoA (4). Scd enzyme activity increases 20–100-fold during the differentiation of 3T3-L1 preadipocytes, primarily due to increased transcription of the scd1 gene (5).

Transcriptional regulation of late adipocyte development has been well characterized. CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferator activated receptor γ2 (PPARγ2) are critical transcription factors required for terminal differentiation of preadipocytes. They have been shown to regulate the transcription of several adipocyte-specific genes (6–8). C/EBPα has been shown to bind to the 5′-flanking region of the scd1 gene and to activate scd1 mRNA expression during late adipocyte differentiation (8). Transcriptional events during the early differentiation program are more poorly characterized.

Cyclic AMP may be a major factor involved in transcriptional regulation during early preadipocyte differentiation. An increase in intracellular cAMP concentrations during early differentiation is required for optimal adipose conversion in 3T3-L1 preadipocytes. Two eukaryotic transcription factors, characterized in other systems, are known to be important regulators of gene expression in response to cAMP. cAMP response element binding protein (CREB) is activated by cAMP through protein kinase A or calcium/calmodulin-dependent protein kinase to activate the expression of many genes (9). Activator protein 2 (AP-2), a less well characterized factor, stimulates gene expression through activation either by cAMP or phorbol ester-induced signal transduction (10).

In the present study, cAMP was shown to increase scd1 mRNA expression in preadipocytes and during the early stages of 3T3-L1 differentiation. During differentiation, scd1 was expressed at two distinct times. An early induction was caused by cAMP working through genetic response elements within the 5′-flanking region of the scd1 gene. A late induction occurred just prior to accumulation of triacylglycerides and was independent of cAMP. The early induction represents a novel event...
of preadipocyte differentiation. It is one of the few and earliest events linked to cAMP action, a molecule required for the triggering of differentiation of 3T3-L1 preadipocytes. The induction also provides a novel early marker for studying the stages of adipocyte development.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and other nucleic acid modifying enzymes were obtained from Promega. Radiolabeled deoxyribonucleic acids (DNA) were from DuPont NEN. 8-Br-cAMP, forskolin, 1,9-dideoxyforskolin, and dexamethasone were from Sigma. G418, lipofectamine transfection reagent, and tissue culture supplies were from Life Technologies, Inc. Mutagenesis was performed using an *in vitro* mutagenesis kit from Bio-Rad. Methylation of cytosine residues was obtained from Aldrich. Insulin was from Lilly. The reporter gene plasmids pCAT(An) and pTKCAT(An) were gifts from Howard Towle, University of Minnesota. Oligonucleotide primers were chemically synthesized in the Department of Biochemistry at the University of Wisconsin and purified in our laboratory or were from Eppendorf Scientific or Operon Technologies.

**Cell Culture**—3T3-L1 preadipocytes were maintained and induced to differentiate as described (11).

**Isolation of RNA and Quantitation of scd1 mRNA Levels**—RNA was isolated from 3T3-L1 cells as described by Chirgwin et al. (12). RNase protection analysis was performed according to Melton et al. (13). Antisense RNA probes were synthesized using T7 RNA polymerase, HinfIII-linearized pKR 180 plasmid templates (14), and [α-32P]CTP. 500,000 cpm of probe were hybridized to 15 μg of RNA at 54°C in 50% formamide for 16 h followed by digestion with RNases A and T1. The protected probe fragments were analyzed on 6–8% denaturing acrylamide sequencing gels and were visualized by autoradiography. Gels were quantitated by laser densitometric scanning of autoradiograms.

**RESULTS**

**scd1 Expression Is Induced during Early Differentiation**—scd1 is known to be expressed at high levels during the late stages of differentiation, appearing after 3 days and peaking after 5 days (5, 20). Prior studies on the induction of scd1 have never examined early differentiation time points for expression.

Cells were treated with MDI and harvested at various time points during the course of differentiation. Total RNA was collected and analyzed by RNase protection using an scd1-specific complementary riboprobe. mRNA was used as a control for nonspecific hybridization. B, data for A were quantitated and plotted as fold induction of differentiated time points over undifferentiated cells. C, preadipocytes were harvested after 0.25, 0.5, 1, 2, 4, 8, 24, 32, and 48 h of differentiation. Total RNA was collected and analyzed by Northern blot analysis probed with pAL15 cDNA.
pAL15 (21), a cDNA probe generated from 3T3-L1 preadipocytes (5), was also tested to determine if the early induction of scd1 corresponded to a global induction of gene expression or mRNA stabilization. pAL15 remained unchanged at all time points within the early induction period in response to differentiation (Fig. 1B). pAL2 mRNA, a fatty acid binding protein known to be expressed during late differentiation, was not expressed during the first 2 days of differentiation (data not shown). We know of no other genes that are expressed during two distinct stages of preadipocyte differentiation.

**cAMP Activates scd1 Expression in 3T3-L1 Preadipocytes**—Methylisobutylxanthine (MIX), dexamethasone, and insulin are required for differentiation of 3T3-L1 preadipocytes. Treatment of cells with insulin and dexamethasone had no effect on scd1 mRNA levels (data not shown). To determine if the induction of scd1 during early differentiation resulted from an increase in cAMP caused by MIX, 3T3-L1 preadipocytes were grown until 2-day postconfluence and treated with compounds known to raise intracellular cAMP concentrations. Cells were treated for 12 h with fetal bovine serum and 1 mM 8-Br-cAMP, a cell-permeable cAMP analog, 115 μg/ml MIX, 50 μM forskolin, an activator of adenylyl cyclase, or 50 μM 1,9-dideoxyforskolin, a forskolin analog unable to activate adenylyl cyclase. Cells were harvested, and total RNA was collected and subjected to RNase protection analysis (Fig. 2A). 8-Br-CAMP, MIX, and forskolin all caused an increase in scd1 mRNA over preadipocytes exposed to vehicle alone. 1,9-Dideoxyforskolin had no effect.

To determine the time dependence of cAMP induction on scd1 mRNA, cells were treated with 1 mM 8-Br-cAMP for various lengths of time before assaying by RNase protection. scd1 mRNA became detectable after 1 h of cAMP treatment, reaching a maximum induction of 9.1-fold by 6 h (Fig. 2B). Additionally, cells were treated with cycloheximide, a protein synthesis inhibitor, for 1 h prior to and concurrent with a 12-h 8-Br-cAMP treatment. Cycloheximide-treated cells showed a 75% decrease in scd1 mRNA compared with cells treated for 12 h with 8-Br-cAMP alone (Fig. 2B). This demonstrates that ongoing protein synthesis was required for maximal scd1 mRNA induction by cAMP.

To determine if the increase in scd1 mRNA was a result of increased transcription of the scd1 gene, cells were treated with 8-Br-cAMP for 6 h and assayed by nuclear run-on transcription assay. Labeled probes from the treated and untreated preadipocytes were hybridized to membranes containing DNA for pGEM, a negative control, pAL15, a clone whose expression does not change in response to cAMP treatment, and scd1. Treated cells showed an 8.8-fold induction in scd1 transcript with no induction in pAL15 transcript compared with untreated preadipocytes (Fig. 2C). These results demonstrated that the induction of scd1 by cAMP was caused by increased scd1 transcription.

**cAMP Induction of scd1 Expression Is Preadipocyte-specific**—To determine if cAMP induction of scd1 expression occurred in mature adipocytes, day 6 differentiated cells were treated with 1 mM 8-Br-cAMP for the indicated lengths of time and then assayed by RNase protection. Basal scd1 expression was high in mature adipocytes, as expected, because adipocytes are actively engaged in desaturation as part of their processing and storage of triacylglycerides. However, in the presence of cAMP, scd1 mRNA levels were not induced in the mature adipocytes during any of the time exposures (Fig. 3).

**Analysis of the 5′-Flanking Region of the scd1 Gene**—Constructs containing regions of the 5′-flanking region from −4300, −600, −363, −253, −170, −76, and −40 to +30 were cloned into the pCAT(An) reporter expression system. Chimeric reporter constructs were transiently transfected into 3T3-L1 preadipocytes and assayed for CAT expression in response to 1 mM 8-Br-cAMP treatment for 12 h. The −253 construct gave a 7.2-fold increase of CAT expression over untreated cells (Fig. 4A). The larger constructs did not show higher induction than the −253 construct, indicating that the cAMP response was contained within the nucleotides from −253 to +30 of the scd1 gene. The −76 and −40 constructs showed a smaller induction.
in response to cAMP treatment (Fig. 4A). The region from −76 to +30 appears to contain only a portion of the response, suggesting the presence of multiple cAMP-responsive elements. (RSV)CAT vector, containing a constitutively active promoter, and the pCAT(An) vector alone showed no induction in response to cAMP.

Role of Trans-acting Factors in the Induction of scd1 by cAMP—CREB and AP-2 are the two identified, and most common, transcription factors responsible for mediating cAMP induction of gene expression (9). Both CREB and AP-2 interact through specific DNA sequences. The −253 to +30 region of the scd1 5′-flanking region does not contain any CREB response elements but contains several potential AP-2 binding sites. Potential sites were identified by sequence comparison to previously identified AP-2 response elements (Fig. 5A). C/EBPα is a transcription factor known to bind to the scd1 5′-flanking region between bases −60 and −80 (8). C/EBP family members also have been shown to mediate cAMP responsiveness in other genes (22).

AP-2 has been characterized as a transcription factor that alters gene expression in response to cAMP and phorbol ester (10). To test the involvement of AP-2 on scd1 expression, stably transfected −253 pCAT(An) preadipocytes were treated with 115 μg/ml MIX alone for 6 h and with phorbol 12-myristate 13-acetate (PMA) alone for 1 h at indicated concentrations. Also, cells were treated with MIX for 6 h with PMA added during the last hour at indicated concentrations. PMA-treated cells showed no increase in CAT expression over untreated preadipocytes. However, cells treated with MIX and PMA showed a dose-dependent increase in CAT expression over cells treated with MIX alone (Fig. 5B). The additional induction with phorbol ester suggests a role for AP-2 in the activation of scd1 mRNA expression.

To further establish the role of AP-2 in the induction of scd1 mRNA expression by cAMP, the potential AP-2 response elements were characterized. The potential AP-2 response elements highlighted in Fig. 5A were cloned into pTKCAT(An) reporter construct and transiently transfected into 3T3-L1
Differentiating preadipocytes drive the early induction of reporter constructs. 3T3-L1 preadipocytes were stably transfected with the −253 to +30 region of the scd1 gene cloned into the pCAT(An) reporter system. Cells were harvested after various time points of differentiation and assayed for CAT activity. Data are presented as fold induction of CAT activity from differentiating cells over preadipocytes.
expression systems using the scd1 promoter or a heterologous promoter. Deletion analysis suggests that multiple cAMP response elements are located within the −253 to +30 promoter sequence. Constructs containing the region from −253 to −76 and from −76 to +30 both respond to cAMP, confirming the presence of multiple response elements. Both regions contain potential AP-2 binding sites, although only the site from the −22 to −11 region confers cAMP responsiveness to a reporter gene. PMA treatment is able to enhance the induction observed with cAMP. The dual phorbol ester and cAMP responsiveness, along with the similarity of the −22 to −11 region to a known AP-2 response element, suggests that AP-2 is a mediator of cAMP induction of scd1 expression. A C/EBP binding site is also present within the −253 to +30 region. cAMP has been shown to stimulate acetyl-CoA carboxylase expression through a C/EBP isoform in differentiating 3T3-A5 preadipocytes (22).

Mutagenesis of the C/EBP binding site from the scd1 gene does not affect cAMP induction, ruling out the requirement of C/EBP family members for the induction of scd1 during early differentiation. An additional, unknown factor that interacts with the −250 to −76 region is probably required to obtain the full cAMP effect.

cAMP-induced gene expression through AP-2 or CREB typically occurs within seconds to minutes after stimulation and is usually a cycloheximide-independent effect (9). The induction of scd1 requires several hours of cAMP exposure and is negated upon treatment with cycloheximide. This evidence rules out a direct effect of cAMP and AP-2 on scd1 expression and suggests a more complicated regulation. cAMP-induced signal transduction may be activating an unknown gene through AP-2, CREB, or an unidentified factor. The unknown protein may act to allow AP-2 induction of scd1 expression. This factor cannot be the AP-2 protein itself because AP-2 is present in preadipocytes without cAMP stimulation.2 The factor may be a protein needed for AP-2 activation. The treatment of stably transfected preadipocytes with phorbol ester provides evidence for the indirect mechanism of scd1 induction. Treatment of preadipocytes with phorbol ester alone does not cause scd1 induction. PMA only induces scd1 expression after the cells have been treated with cAMP for several hours. Along with the cycloheximide data, this suggests that a protein is made in response to several hours of cAMP treatment, allowing for eventual activation of AP-2 by phorbol ester.

scd1 is induced during preadipocyte differentiation at two separate stages. There are three pieces of evidence demonstrating that the two inductions are distinct regulatory events. First, the early induction of scd1 is driven by cAMP. cAMP does not affect scd1 in fully differentiated adipocytes, the time of maximal late scd1 expression. cAMP-elevating agents are used to differentiate preadipocytes and are removed from the adipogenic mixture after 48 h. Therefore, after day 2, intracellular cAMP concentrations are returned to basal levels and cannot act to stimulate scd1 during the late induction. Second, chimeric CAT gene reporter constructs linked to the scd1 CAMP-responsive region are activated by differentiating cells during the period of early induction but are not active during the later stage of differentiation. Third, prostaglandin F2α has been shown to inhibit the differentiation of 3T3-L1 preadipocytes (25, 26). Prostaglandin F2α-inhibited cells do not express late-induced scd1 mRNA (25) but leave the early induction intact (26). These results demonstrate that the early induction caused by cAMP can be separated from the late effect. The transcriptional machinery responsible for the early scd1 induction must be inactive during later differentiation. The early and late inductions are generated through separate mechanisms and probably play distinct roles in the differentiation of preadipocytes.

The early induction of scd1 by cAMP provides a new marker for the analysis of preadipocyte differentiation. Upon stimulation of differentiation, c-fos, c-jun, and c-myc expression peaks and decrease within the first 5 h (2). At approximately 24 h, the cells undergo DNA replication, followed by cell division. The CAMP response provides a differentiation marker that lies between these two events and acts as a unique indicator for cAMP action within the 1st day of 3T3-L1 differentiation.

The early induction of scd1 expression occurs several days prior to fat accumulation by the cells. The cellular purpose of Scd during this time is probably independent of its lipogenic role during triacylglycerol storage. Scd is responsible for producing monounsaturated fatty acids from saturated precursors. The utility of this function during early differentiation could serve several purposes. Scd may be up-regulated to alter membrane dynamics. Increased unsaturated fatty acid within membranes alters fluidity and may allow for altered protein binding, conformation, and function. Scd may also be used for an early enzymatic step in the synthesis of fatty acid-like signaling molecules. Fatty acids and fatty acid derivatives are increasingly being recognized as intermediate signals in many cellular events (27). The early induction may be a prerequisite for the production of a signaling molecule needed for the later stages of differentiation. For example, work on the peroxisome proliferator activated receptor γ2 (PPARγ2) has shown that forced expression of PPARγ2 and administration of a PPARγ2 ligand can stimulate adipogenesis in NIH-3T3 fibroblasts (7, 28). A similar effect is observed when MIX is substituted for PPARγ2 ligand (7), suggesting that cAMP signals the synthesis of the endogenous PPARγ2 ligand. PPAR ligands are known to be fatty acid-like molecules (29). This report offers the first example of a CAMP-stimulated, fatty acid-modifying enzyme involved in adipocyte differentiation and may be important to the first complete understanding of cell signaling during adipocyte conversion.

Acknowledgments—We thank Carolyn Miller for helpful discussions and manuscript reviews, Katrina Waters and Tetsaka Takova for helpful discussions, and Debbie Czarniecki for technical advice.

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