Transient Induction of ENC-1, a Kelch-related Actin-binding Protein, Is Required for Adipocyte Differentiation*

Ling Zhao, Francine Gregoire‡, and Hei Sook Sul§

Received for publication, January 10, 2000

From the Department of Nutritional Sciences, University of California, Berkeley, California 94720

The differentiation of preadipocytes to mature adipocytes involves striking morphological and biochemical changes. Fibroblastic preadipocytes stop dividing, undergo differentiation, and attain the biochemical profiles and rounded, lipid-filled morphology typical of mature adipocytes. Both established preadipocyte cell lines such as 3T3-L1 cells and primary cultures of adipose-derived stroma-vascular precursor cells have been used in examining the process of adipogenesis in vitro. Adipocyte differentiation is induced by adipogenic mixture, typically a combination of the synthetic glucocorticoid dexamethasone and methylisobutylxanthine (MIX) and of 3T3-L1 cells. Treatment with the adipogenic inducers dexamethasone and methylisobutylxanthine causes an increase in ENC-1 mRNA levels specifically in preadipocytes, and methylisobutylxanthine is the main effector of ENC-1 expression. The induction of ENC-1 precedes expression of the transcription factors, peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding protein (C/EBPα), and other adipocyte markers including adipocyte fatty acid-binding protein. The ENC-1 induction correlates with the subsequent differentiation of primary stroma-vascular cells into adipocytes. Furthermore, decreasing the endogenous ENC-1 levels by stable antisense transfection, thereby preventing the transient induction, effectively inhibits 3T3-L1 adipocyte differentiation. Overall, these studies indicate that ENC-1, an actin-binding protein, plays a regulatory role early in adipocyte differentiation when cytoskeletal reorganization and cell shape change from fibroblastic preadipocytes to spherical adipocytes occur.

The differentiation of preadipocytes to mature adipocytes involves striking morphological and biochemical changes. Fibroblastic preadipocytes stop dividing, undergo differentiation, and attain the biochemical profiles and rounded, lipid-filled morphology typical of mature adipocytes. Both established preadipocyte cell lines such as 3T3-L1 cells and primary culture of adipose-derived stroma-vascular precursor cells have been used in examining the process of adipogenesis in vitro. Adipocyte differentiation is induced by adipogenic mixture, typically a combination of the synthetic glucocorticoid dexamethasone and the phosphodiesterase inhibitor methylisobutylxanthine (MIX) in the presence of fetal bovine serum. Subsequent to this treatment, cells start to change shape and undergo postconfluent mitoses, and subsequently the majority of the cells attain adipocyte phenotype in 5–7 days (1, 2). Adipocyte differentiation involves changes in the levels of more than 100 proteins (3). Those proteins involved in the specialized metabolic role of mature adipocytes, including lipid transport and metabolism and hormone responsiveness, are induced (1, 2). It is now well established that two families of transcription factors, peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer binding protein (C/EBPα), are induced early in adipocyte differentiation and activate adipocyte genes and thereby promote adipogenesis (4–6).

Whereas these adipogenic transcription factors have been extensively studied in recent years, less attention has been focused on the morphological alteration during adipocyte differentiation. The type and level of extracellular matrix components secreted (7–10) and cytoskeleton components (11) are altered during adipocyte differentiation. For example, expression of actin and tubulin and cytoskeleton assembly decrease as cells change from a fibroblastic to a spherical shape of adipocytes (11). The down-regulation of the actin and tubulin precedes the overt changes in morphology and the expression of adipocyte-specific gene expression (11). These changes in cell shape reflect a distinct process in differentiation and are not the result of lipid accumulation. Cells induced to differentiate in the absence of biotin, a cofactor for lipogenesis, or in the presence of lipolytic agents do not accumulate lipids but still undergo biochemical and morphological differentiation (12). Furthermore, the shape change from fibroblastic preadipocytes to rounded mature adipocytes, determined by the changes in cytoskeletal organization and/or contacts with extracellular matrix, is required for adipocyte differentiation. Maintaining 3T3-F442A preadipocytes on fibronectin matrices, non-hydrated, non-deformable extracellular matrices or highly charged molecules like polylysine prevented not only the cell shape changes but also the expression of lipogenic enzymes, resulting in a decrease in triacylglycerol accumulation (13, 14). Disrupting actin filaments by cytochalasin D treatment could overcome the inhibitory effect of fibronectin (13). Differentiation of 3T3-F442A cells maintained in regular culture dishes was also promoted by the cytochalasin B treatment perhaps by allowing the necessary cytoskeletal remodeling. Moreover, with long term exposure, even non-differentiating 3T3-C2 cells underwent morphological and biochemical adipocyte differentiation (15). These studies suggest that cytoskeletal remodeling that accompanies the cell shape change is a distinctive process and is a prerequisite for adipocyte differentiation. The regulatory molecule(s) that are involved in this cytoskeletal reorganization during adipocyte differentiation have been largely unidentified to date.
Here we report the identification of ENC-1 by differential display of mRNA prepared from the primary culture of the stroma-vascular fraction of adipose tissue. ENC-1 is a kelch-related actin-binding protein and has been previously identified to be expressed in a brain-specific manner (16). We found that ENC-1 is expressed in adipose tissue, specifically in the adipose-derived stroma-vascular fraction. ENC-1 is transiently induced early during the hormonal induction phase of the adipocyte differentiation of primary cells from the adipose-derived stroma-vascular fraction and of 3T3-L1 cells. The transient induction of the ENC-1 precedes expression of the transcription factors, PPARγ and C/EBPα, and other adipocyte-specific markers including adipocyte fatty acid-binding protein (aFABP). We found that the ENC-1 induction correlates with the degree of differentiation of primary preadipocytes in culture and that decreasing the endogenous ENC-1 levels by stable antisense transfection thereby preventing transient induction greatly inhibits 3T3-L1 adipocyte differentiation. Overall, these studies indicate that ENC-1 plays a regulatory role early in adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Preparation of the Stromal-Vascular Fraction from Rat Adipose Tissue**—The adipose-derived stroma-vascular fraction from rats was prepared as described previously (17). Briefly, the subcutaneous inguinal fat deposits from female Zucker rats were dissected under sterile conditions, and the lymph nodes were carefully removed. The stroma-vascular cells were obtained from the minced fat tissues by collagenase (Sigma, 540 units/mg) digestion at 1 mg/ml at 37 °C for 45 min in Heps-phosphate buffer (10 mM HEPES, pH 7.4, 135 mM NaCl, 2.2 mM CaCl₂, 1.25 mM MgSO₄, 0.45 mM KH₂PO₄, 2.17 mM Na₂HPO₄, 5 mM g-glucose, and 2% v/v bovine serum albumin) under agitation. The resulting cell suspension was homogenized by pipetting with a 10-ml serological plastic pipette, filtered through a 100-μm nylon filter, and centrifuged at 400 × g for 10 min. The floating top layer of mature adipocytes was removed, the pellets were washed several times, and the suspended cells were filtered through a 25-μm nylon filter to eliminate residual aggregates. After examination for viability and counting, the cells were plated at a density of 2.5 × 10⁶ cells/cm² into 100-mm culture dishes (Falcon) in the presence of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. When the cells reached confluence, the differentiation was initiated by the addition of 0.1 μM dexamethasone, 0.25 mM MIX, and 17 mM insulin in DMEM supplemented with 10% fetal bovine serum. After 48 h, the differentiation medium was replaced by DMEM supplemented with 10% fetal bovine serum plus insulin only. 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% fetal bovine serum. For differentiation, confluent cells were treated with 1 μM dexamethasone/0.5 mM MIX for 72 h, after which the differentiation medium was replaced with DMEM containing 10% fetal bovine serum.

**Stable and Transient Transfections**—For stable transfection, 3T3-L1 cells were plated at 40% confluence the day before transfection. 20 μg of ENC-1 antisense plasmid per 100-mm² dish was transfected into 3T3-L1 cells by the calcium phosphate/DNA co-precipitation method. COS cells were plated at a density of 2.5 × 10⁴ cells/cm² into 100-mm² dishes (Falcon) in the presence of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml of Moloney murine leukemia virus reverse transcriptase, and individual clones were isolated into 24-well plates, expanded, and individually confirmed by restriction mapping and sequencing. The antisense ENC-1 construct was also prepared by PCR from the ENC-1 cDNA clone confirmed by restriction mapping and sequencing. The antisense construct was confirmed by restriction mapping.

**Isolation of Total RNA and Northern Blot Analysis**—The rat tissues were quickly frozen in liquid nitrogen and were homogenized in the presence of 4 ml guanidine isothiocyanate plus β-mercaptoethanol. Total RNA was isolated by cesium chloride ultracentrifugation. Cell monolayers were washed twice with PBS; total RNA was harvested in TriZOL reagent (Life Technologies, Inc.). Total RNA was isolated according to the instructions of the manufacturer. The indicated amount of total RNA from tissues of rat or from cells was electrophoresed in 1% formaldehyde-agarose gel in 2.2 M formaldehyde, 20 mM MOPS, 1 mM EDTA, stained with ethidium bromide, and transferred to Hybond N+ nylon filter to eliminate residual aggregates. After examination for viability and counting, the cells were plated at a density of 2.5 × 10⁶ cells/cm² into 100-mm culture dishes (Falcon) in the presence of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. When the cells reached confluence, the differentiation was initiated by the addition of 0.1 μM dexamethasone, 0.25 mM MIX, and 17 mM insulin in DMEM supplemented with 10% fetal bovine serum. After 48 h, the differentiation medium was replaced by DMEM supplemented with 10% fetal bovine serum plus insulin only. 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% fetal bovine serum. For differentiation, confluent cells were treated with 1 μM dexamethasone/0.5 mM MIX for 72 h, after which the differentiation medium was replaced with DMEM containing 10% fetal bovine serum.

**Differential Display**—Differential display was performed using RNAmag Kit (GeneHunter) essentially as described (18). Briefly, total RNA from primary cultured cells at day 0, 12 h, 36 h, and day 9 was ana-
Identification and Characterization of ENC-1—To identify novel gene(s) that are differentially expressed in early stages of adipocyte differentiation and that may play an important role in initiating and/or maintaining adipocyte phenotype, we employed differential display techniques. RNA was prepared from primary culture of rat adipose-derived stroma-vascular cells. We employed primary culture, which may reflect in vivo physiological conditions better than the established preadipocyte lines. At confluence, the differentiation of primary preadipocytes to adipocytes was induced by treatment with 0.1 μM dexamethasone, 0.25 mM MIX, and 17 nM insulin in DMEM supplemented with 10% fetal bovine serum and 17 nM insulin only. The total RNA was harvested at day 0, 12 h, 36 h, and day 9 of the differentiation. The total RNA from rat liver and muscle was also prepared and used as a negative control for the differential display. Two candidates that were highly expressed at 12 and 36 h after treatment with the adipogenic mixture but at a low level at days 0 and 9 (fully differentiated adipocytes) and undetectable in liver and muscle were further examined. We report herein one such cDNA sequence to a 4.8-kb mRNA. Because Northern blot analysis revealed that the corresponding RNA was most abundantly expressed in brain, a mouse brain cDNA AZAP library was screened for a full-length cDNA sequence. We isolated and fully sequenced a cDNA clone with an insert size of 9394 bp containing an open reading frame of 1767 bp in length. 4718 bp containing an open reading frame of 1767 bp in length. 4718 bp containing an open reading frame of 1767 bp in length.

RESULTS AND DISCUSSION

ENC-1 in Adipocyte Differentiation

identification and Characterization of ENC-1—To identify novel gene(s) that are differentially expressed in early stages of adipocyte differentiation and that may play an important role in initiating and/or maintaining adipocyte phenotype, we employed differential display techniques. RNA was prepared from primary culture of rat adipose-derived stroma-vascular cells. We employed primary culture, which may reflect in vivo physiological conditions better than the established preadipocyte cell lines. At confluence, the differentiation of primary preadipocytes to adipocytes was induced by treatment with 0.1 μM dexamethasone, 0.25 mM MIX, and 17 nM insulin in DMEM supplemented with 10% fetal bovine serum for 48 h, after which the cells were maintained in DMEM containing 10% fetal bovine serum and 17 nM insulin only. The total RNA was harvested at day 0, 12 h, 36 h, and day 9 of the differentiation. The total RNA from rat liver and muscle was also prepared and used as a negative control for the differential display. Two candidates that were highly expressed at 12 and 36 h after treatment with the adipogenic mixture but at a low level at days 0 and 9 (fully differentiated adipocytes) and undetectable in liver and muscle were further examined. We report herein one such cDNA sequence to a 4.8-kb mRNA. Because Northern blot analysis revealed that the corresponding RNA was most abundantly expressed in brain, a mouse brain cDNA AZAP library was screened for a full-length cDNA sequence. We isolated and fully sequenced a cDNA clone with an insert size of 4718 bp containing an open reading frame of 1767 bp in length. A 5'-noncoding region of 0.4 kb and 3'-noncoding region of 2.5 kb were present. The polyadenylation signal of AATAAA was present at nucleotide 4690. Subsequent to our cloning and sequencing, a GenBank™ search revealed that this gene had been cloned by PCR amplification as a brain-specific sequence and named ENC-1 (16). Although the coding region of our clone is exactly the same as the published ENC-1 sequence, our clone had an additional 0.18 kb at the 5'-noncoding and 2.3 kb at the 3'-noncoding regions representing a complete cDNA sequence. ENC-1 shares an approximately 50% amino acid sequence homology with Drosophila kelch, a component of ring canals that connect the nurse cells with the developing oocyte in egg chambers (19). Like kelch, ENC-1 was shown to be associated with F-actin in transfected medulloblastoma Daoy cells (16). However, unlike Kelch containing two open reading frames in a single transcript (19), ENC-1 contains only one open reading frame. To confirm the protein encoded by the ENC-1 cDNA sequence, we transiently transfected COS cells with an expression plasmid containing the full-length ENC-1 tagged with a HA epitope at the C terminus. As shown in Fig. 1A, Western blot analysis of the cell lysates detected a protein with an apparent molecular mass of 66 kDa, which is the predicted size of full-length ENC-1 plus a HA tag. We also stably transfected ENC-1 into 3T3-L1 cells and examined subcellular localization of ENC-1 by immunofluorescence. As shown in B, ENC-1 was colocalized with F-actin in 3T3-L1 preadipocytes, suggesting its function as an actin-binding protein in the context of adipocyte differentiation. Similar to kelch, in addition to the C-terminal kelch repeat domain, ENC-1 contains an N-terminal BTB/POZ domain. While the kelch repeat domain is implicated in mediating the interaction with actin filaments (20–22), the BTB/POZ domain, as in some of the zinc finger proteins, defines a conserved protein-protein interaction interface for either homodimeric or heterodimeric formation (23–25). ENC-1 may interact with other BTB/POZ domain-containing proteins, either as a homodimer or by interaction with a yet to be identified protein, ENC-1 may bundle or cross-link actin filaments resulting in actin cytoskeleton remodeling.

ENC-1 Is Expressed in Adipose Tissue but Only in the Stromal-Vascular Fraction—ENC-1 was cloned from the primary culture of stroma-vascular cells isolated from adipose tissue. As shown in Fig. 2A, Northern blot analysis indicates a significant but low level of ENC-1 mRNA in adipose tissue. ENC-1 mRNA was highly expressed in brain and at a low level in testis but not in other tissues, such as adrenal, spleen, kidney, liver, muscle, heart, lung, and intestine. Because ENC-1 mRNA in adipose tissue was lower than that observed in brain and because adipose tissue is comprised of mature fat cells together with the stroma-vascular fraction, we separated the adipocyte fraction from the stroma-vascular fraction. As shown in Fig. 2B, mRNA for PPARY, an adipocyte transcription factor, was found at a high levels in the mature adipocyte fraction but was barely detectable in the stroma-vascular fraction. In contrast, ENC-1 was predominantly expressed in the stroma-vascular fraction of the adipose tissue at a high level and was barely detectable in the mature adipocyte fraction. This is in accord with the fact that the stroma-vascular fraction is mainly comprised of precursor cells at different stages of adipose development, which we employed for primary culture from which we prepared the mRNA template for differential display. The fact that ENC-1 was abundantly expressed in the stroma-

FIG. 1. Detection of the HA-tagged ENC-1 protein and cellular localization of ENC-1 in stably transfected 3T3-L1 preadipocytes. A, 30 μg of whole cell lysate from COS cells transfected with HA-tagged ENC-1 (lane 1) and vector-transfected control cells (lane 2) were fractionated onto 7.5% SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis using mouse HA monoclonal antibody. The signal was detected by the enhanced chemiluminescence system described under “Experimental Procedures.” Markers in kDa are indicated. B, 3T3-L1 cells stably transfected with HA-tagged ENC-1 were fixed and permeabilized as described under “Experimental Procedures.” ENC-1 was localized by immunofluorescence using mouse HA monoclonal antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse Ig. In the same cell, actin filaments were visualized by rhodamine-labeled phalloidin. The bar represents 20 μm.
ENC-1 Is Induced in Primary Culture of Stroma-Vascular Cells upon Treatment with Adipogenic Agents, and ENC-1 Induction Correlates with Subsequent Adipose Conversion—In light of the differential expression pattern in the two fractions of adipose tissue, we examined ENC-1 expression during differentiation of primary culture of stroma-vascular cells into adipocytes. Consistent with the fact that we identified ENC-1 by differential display as a sequence that is highly induced early during differentiation, as shown in panel B of Fig. 3, the level of ENC-1 mRNA increased 12-fold as early as 12 h upon the adipogenic treatment of 0.1 μM dexamethasone, 0.25 mM MIX, and 17 nM insulin, and the level remained high through 36 h. mRNAs for adipocyte markers, PPARγ and aFABP, on the other hand, were not detectable at confluence or at 12 h upon dexamethasone/MIX/insulin treatment, and by 36 h, the expression of adipocyte markers was induced and easily detectable. At day 9 when the majority of the cells had differentiated into fat cells, as judged by cell morphology of rounded lipid-filled and high levels of expression of PPARγ and aFABP, ENC-1 mRNA was not detectable. The decrease in ENC-1 mRNA levels observed in in vitro differentiated adipocytes of primary cells is consistent with the fact that ENC-1 is predominantly expressed in the stroma-vascular fraction but only barely detectable in the mature adipocyte fraction from fat pads. As shown in panel A of Fig. 3, we also examined a preparation of primary cells that did not show the early transient induction of ENC-1. Because of the nature of the primary culture, the preadipocytes from primary culture vary in their response to the differentiation treatment. For example, the differentiation of primary cells has been shown to be dependent on the donor of the tissue and the age of the donor (17, 26, 27). Comparison of these two different preparations of the primary culture designated A (minimal differentiation) and B (full differentiation) provided us with insight into the role of the transient induction of ENC-1. Both preparations were treated in the exactly the same manner. As shown in Fig. 3, whereas cells in panel B showed a 80–90% differentiation, reflected by higher levels of PPARγ and aFABP mRNA, the cells in panel A showed poor differentiation, reflected by the absence of increases in PPARγ and aFABP mRNA levels. Whereas the photomicrograph of the Oil Red O-stained preadipocytes of panel B at day 9 showed rounded lipid-filled adipocytes, the cells in panel A at day 9 showed fibroblastic morphology with no significant lipid staining, further confirming the differences in the degree of the differentiation (Fig. 3). As indicated above, whereas cells in panel B showed 12-fold induction of ENC-1, the cells in panel A showed barely detectable levels of ENC-1 at all time points examined. This indicates that transient early induction of ENC-1 in primary preadipocytes in culture predicted the subsequent adipose conversion of these cells.

Time Course of the ENC-1 Expression during 3T3-L1 Adipocyte Differentiation—To further examine the potential role of ENC-1 in adipocyte differentiation, we studied the time course of ENC-1 expression during 3T3-L1 adipocyte differentiation. As shown in Fig. 4, upon treatment with 1 μM dexamethasone, 0.5 mM MIX, the levels of ENC-1 mRNA increased significantly at 20 h, continued to increase at 40 h, and reached the maximum of approximately 8-fold at day 3. After the replacement of the differentiation medium with DMEM containing 10% fetal bovine serum only, ENC-1 mRNA levels decreased sharply back to the basal level at day 4 and remained at the low level throughout the remaining period of differentiation. The kinetics of ENC-1 expression were quite different from those of differentiation-related genes including aFABP. As shown in Fig. 4, aFABP mRNA was undetectable until 40 h upon differentiation, but the levels increased as cells progressed through the differentiation program. Clearly, the maximal induction of ENC-1 during differentiation is earlier than those of the adipocyte-specific markers including aFABP (Fig. 4) and the transcription factors, PPARγ and C/EBPα (shown in Fig. 6), that are known to activate adipocyte genes, suggesting that the

vascular fraction but not in the mature fat cell fraction from adipose tissue also suggests that ENC-1 might play a role early in the development of adipose tissue in vivo.
stage when ENC-1 elicits its function, if it does, is earlier than those for PPARγ and C/EBPα.

MIX Induces ENC-1 Expression Selectively during Adipocyte Differentiation—Standard protocol for 3T3-L1 adipocyte differentiation involves treatment with dexamethasone and MIX in the presence of fetal bovine serum. However, the target genes whose expression are modulated by each of these agents are far from being identified to date. Because the induction of ENC-1 was detectable early upon the dexamethasone/MIX treatment, we examined the effects of each of these agents on ENC-1 mRNA levels. Similar to the results shown in Fig. 4, treatment with the dexamethasone and MIX in combination for 24 h caused ENC-1 induction. Furthermore, MIX alone induced the same degree of the ENC-1 expression as that by dexamethasone/MIX treatment. Treatment with dexamethasone alone did not show any significant effect on ENC-1 mRNA levels. Clearly, the increase in ENC-1 expression during adipocyte differentiation was brought about by MIX present in the differentiation mixture. To test the specificity of the MIX effect on ENC-1 expression during adipogenesis, we also treated NIH3T3 fibroblasts and C2C12 myoblasts with dexamethasone/MIX as we did for 3T3-L1 cells. As shown in Fig. 5, both of these cell types expressed detectable levels of ENC-1 mRNA. However, unlike in 3T3-L1 cells, in NIH3T3 fibroblasts and C2C12 myoblasts, ENC-1 mRNA levels did not change upon dexamethasone/MIX treatment. These data suggest that the induction of ENC-1 gene expression by MIX may be specific to the adipogenic pathway. It has been well documented that MIX present in the differentiation mixture causes cell shape change in 3T3-L1 cells. Upon MIX treatment, the cells decrease their surface and become spindle-shaped. Interestingly, this change in cell shape correlated with the transient induction of ENC-1, an actin-binding protein during MIX treatment. Hernandez et al. (28) reported that ENC-1 expression was increased during differentiation of SMS-KCNN cells to neural cells causing an extensive network of neurites. Changes in ENC-1 expression during differentiation, whether transient (adipocytes) or persistent (neuronal cells), are consistent with the fact that ENC-1 was expressed in fat tissue as well as brain, implicating its role in the development of these tissues.

Preventing the Transient Induction of ENC-1 by Antisense Transfection Inhibits 3T3-L1 Adipocyte Differentiation—So far, we have demonstrated that ENC-1 was induced early in adipocyte differentiation of primary cells from adipose-derived
stroma-vascular fraction and of 3T3-L1 cells; the transient induction of ENC-1 was earlier than that of transcription factors, PPARy and C/EBPα, and that of terminal adipocyte markers, aFABP; the induction of ENC-1 in primary cells was correlated with the degree of subsequent adipocyte differentiation. To further address the role of ENC-1 expression during adipocyte differentiation, i.e. whether the induction of ENC-1 is required for adipocyte differentiation, we attempted by stable antisense transfection to decrease endogenous ENC-1 expression and to block the transient induction of ENC-1 observed early during differentiation. ENC-1 antisense plasmid was stably transfected into 3T3-L1 cells. Individual stable clones were isolated and screened for their low endogenous ENC-1 expression at confluence. Two antisense clones with the lowest endogenous ENC-1 expression were identified and subjected to the differentiation protocol. Although not shown, both antisense clones gave the same results. Vector-transfected cells were used as controls. The cells were plated at the same density and received the same differentiation treatment at confluence. The cells were harvested at days 0 (at confluence), 3, 4, and 7, and total RNA were prepared. As shown in Northern blot in Fig. 6, the transient increase in ENC-1 mRNA levels was evident at day 3 in control cells. In antisense cells, not only was the ENC-1 mRNA level low at day 0 (at confluence) but also the induction of ENC-1 that normally occurred at day 3 was blocked and ENC-1 mRNA was barely detectable upon dexamethasone/MIX treatment. Furthermore, stable antisense transfection greatly inhibited 3T3-L1 adipocyte differentiation; control cells showed a 90% differentiation, reflected by the high levels of expression of a panel of adipocyte differentiation markers. PPARy and C/EBPα mRNA were barely detectable at day 0 but increased greatly during adipose differentiation. Similarly, other adipocyte genes including lipoprotein lipase, stearoyl-CoA desaturase, fatty acid synthase, and aFABP were induced dramatically during adipocyte differentiation. On the other hand, antisense cells showed only a minimal, if any, degree of differentiation. Increases in mRNA levels for the various adipocyte markers were not detectable even at day 7. No apparent change in control glyceroldehyde-3-phosphate dehydrogenase expression was seen in either antisense or control cells. Similar differences in the degree of adipocyte differentiation was observed as judged by lipid accumulation by Oil Red O staining (data not shown). Interestingly, whereas control cells responded to dexamethasone/MIX at day 2 by forming long and narrow processes, the antisense cells showed no such changes in response to the adipogenic treatment (Fig. 6B). By day 4, control cells became polygonal and started to accumulate lipid droplets, whereas the antisense cells showed no morphological changes that are characteristic of adipocyte differentiation (data not shown). The undifferentiated state of antisense cells persisted to day 8 at which time the control cells clearly showed uniform adipocyte differentiation (Fig. 6B). This antisense experiment clearly demonstrates that the transient induction of ENC-1 is required for subsequent adipocyte differentiation.

At present, we do not know the exact function of ENC-1, whose expression is transiently increased early during adipose conversion. However, we postulate that ENC-1, an actin-binding protein, plays a critical role in dynamic changes that occur in the actin cytoskeleton in response to extracellular signals that trigger adipocyte differentiation. These events lead not only to changes in cell shape, but also to changes in gene expression (29, 30). During adipocyte differentiation, as fibroblastic preadipocytes become spherical in shape, various adipocyte genes (including transcription factors, PPARy and C/EBPα, and terminal markers, aFABP) are induced (2). It has been reported that actin and fibronectin levels decreased somewhat, and this decrease preceded an increase in adipocyte gene expression (11). Furthermore, preventing changes in actin assembly by maintaining the cells on a fibronectin-coated dish blocked adipose conversion of 3T3-F442A cells whereas cytochalasin D reversed this inhibition (13). It has also been shown that dihydrocholesterol B treatment not only efficiently triggered adipose conversion of the primary culture of cells from the stroma-vascular fraction, prolonged treatment caused, albeit at a low level, adipose conversion of even differentiation-defective 3T3-C2 cells (15). The dynamic changes of actin organization probably occur by the temporal and spatial controls of actin assembly/disassembly and cross-linking by various actin-binding proteins. ENC-1 association with actin filaments and its expression pattern during adipogenesis suggest that ENC-1 may be required for reorganization of actin cytoskeleton necessary for cell shape change from fibroblastic to rounded adipocyte morphology that precedes adipocyte differentiation. Further studies addressing the biochemical function of ENC-1 will provide insights into the process of adipocyte differentiation.

REFERENCES

1. Smas, C. M., and Sul, H. S. (1995) Biochem. J. 309, 697–710
2. Gregoire, F. M., Smas, C. M., and Sul, H. S. (1998) Physiol. Rev. 78, 883–899
3. Sidhu, R. S. (1970) J. Biol. Chem. 245, 1111–1118
4. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
5. Lin, F. T., and Lane, M. D. (1992) Genes Dev. 6, 533–544
6. Wang, N. D., Finegold, M. J., Bradley, A., Os, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) Science 269, 1108–1112
7. Dani, C., Doglio, A., Amri, E. Z., Bardon, S., Fort, P., Bertrand, B., Grimaldi, P., and Aliaud, G. (1989) J. Biol. Chem. 264, 10119–10125
8. Aratani, Y., and Kitagawa, Y. (1988) J. Biol. Chem. 263, 16163–16169
9. Weinier, F. R., Shah, A., Smith, P. J., Rubin, C. S., and Zern, M. A. (1989) Biochemistry 28, 4094–4099
10. Antras, J., Hilliou, F., Redziniak, G., and Pairault, J. (1989) Biol. Cell 66, 247–254
11. Spiegelman, B. M., and Farmer, S. R. (1982) Cell 30, 53–60
12. Kuri-Haray, W., Wise, L. S., and Green, H. (1976) Cell 14, 53–59
13. Spiegelman, B. M., and Ginty, C. A. (1983) Cell 35, 657–666
14. Rodriguez Fernandez, J. L., and Ben-Ze’ev, A. (1989) Differentiation 42, 65–74
15. Pairault, J., and Lasni, F. (1987) Biol. Cell 61, 149–154
16. Hernandez, M. C., Andres-Barquin, P. J., Martinez, S., Bulfone, A., Rubenstein, J. L., and Israel, M. A. (1997) J. Neurosci. 17, 3038–3051
17. Gregoire, F. M., Johnson, P. R., and Greenwood, M. R. (1985) Int. J. Obes. Relat. Metab. Disord. 19, 664–670
18. Liang, P., and Pardee, A. B. (1992) Science 257, 967–971
19. Xue, F., and Cooley, L. (1993) Cell 72, 681–693
20. Kim, I. F., Mohammadi, R., and Huang, C. C. (1999) Gene (Amst.) 228, 73–83
21. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
22. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
23. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
24. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
25. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
26. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
27. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
28. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
29. Schmid, M. F., Agris, J. M., Jakana, J., Matsuda, P., and Chiu, W. (1994) J. Biol. Chem. 269, 1108–1112
Transient Induction of ENC-1, a Kelch-related Actin-binding Protein, Is Required for Adipocyte Differentiation
Ling Zhao, Francine Gregoire and Hei Sook Sul

J. Biol. Chem. 2000, 275:16845-16850.
doi: 10.1074/jbc.275.22.16845

Access the most updated version of this article at http://www.jbc.org/content/275/22/16845

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 14 of which can be accessed free at
http://www.jbc.org/content/275/22/16845.full.html#ref-list-1