ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism

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Adipocyte determination and differentiation-dependent factor 1 (ADD1) is a member of the basic helix–loop–helix leucine zipper (bHLH–LZ) family of transcription factors that binds to two distinct DNA sequences and has been associated with both adipocyte development and cholesterol homeostasis (where it has been termed SREBP1). To investigate the biological role of ADD1, we expressed wild-type and dominant negative forms of this protein with retroviral vectors in preadipocytes and nonadipogenic cells. A dominant-negative form of ADD1 with a point mutation in the DNA-binding domain sharply represses the differentiation of 3T3–L1 cells as observed morphologically or by the expression of adipocyte-specific mRNAs. When NIH–3T3 cells ectopically expressing ADD1 are cultured under hormonal conditions not favoring differentiation, they do not overtly differentiate but still activate expression of mRNAs for fatty acid synthase (FAS) and lipoprotein lipase (LPL), two key genes that regulate fatty acid metabolism. Under culture conditions permissive for differentiation including a PPAR activator, 15%–20% of the cells expressing ADD1 undergo adipogenesis while 2%–3% of cells containing a control vector differentiate. Simultaneous expression of ADD1 with PPARγ increases the transcriptional activity of this adipogenic nuclear hormone receptor, suggesting involvement of ADD1 in this pathway. These data indicate that ADD1 plays an important role in fat cell gene expression and differentiation, and suggest that it may function by augmenting a step in PPARγ-mediated transcription.

[*Key Words*: ADD1; bHLH–LZ transcription factor; adipocyte differentiation; dominant negative ADD1]

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Adipocytes are centrally important in the synthesis, storage, and hydrolysis of nutritional energy in the form of triglycerides. Adipocytes store triglycerides during periods of caloric excess and mobilize this reserve when caloric expenditure exceeds intake. To play this role in energy balance, adipocytes possess the enzymes and regulatory proteins needed to carry out de novo lipogenesis, lipoprotein hydrolysis, intracellular fatty acid import, and lipolysis. Most adipocyte differentiation occurs in late prenatal and early postnatal development, and this increase in adipose tissue mass is presumably adaptive in allowing young animals to survive periods of fasting (Salvin 1979; Spiegelman et al. 1993). Adipocytes are derived from relatively undetermined mesenchyme at several anatomical sites. Abnormal regulation of adipocyte differentiation and lipogenesis is linked to important pathological conditions such as obesity, noninsulin-dependent diabetic mellitus (NIDDM), and lipodystrophy (Moller and Flier 1991).

Adipogenesis is a complex process controlled by the interplay of intracellular factors and signals from the environment. During this differentiation, a large number of genes have to be regulated in a selective, coordinated manner, and dramatic changes occur in both cell morphology and gene expression. The availability of established cell lines, including 3T3–F442A and 3T3–L1 preadipocytes, has made it possible to characterize this differentiation program in vitro (Green and Kehinde 1974; Green and Kehinde 1976). Several transcription factors have been linked to the adipocyte differentiation. Considerable evidence has accumulated to suggest that both CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) function as important regulators of adipocyte gene expression and differentiation. C/EBPα is one member of the C/EBP family that also includes C/EBPβ and C/EBPδ (Birkenmeier et al. 1989; Cao et al. 1991). C/EBPα contains a basic leucine zipper domain and *trans*-activates the promoters of several adipocyte-specific genes (Christy et al. 1989; Friedman et al. 1989; Herrera et al. 1989; Kaestner et al. 1990; Park et al. 1990). Whereas C/EBPα is not adipose-specific in vivo, it is induced during adipogene-

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sis. Extensive studies from several groups have demonstrated that overexpression of C/EBPα stimulates adipocyte differentiation in 3T3–L1 cells and C/EBPα antisense mRNA can block 3T3–L1 differentiation (Samuelsson et al. 1991; Umek et al. 1991; Lin and Lane 1992). Retroviral expression of C/EBPα induces adipocyte differentiation in several types of fibroblasts or preadipocytes (Freytag et al. 1994; Lin and Lane 1994). Most recently, it has been shown that C/EBPβ can also promote differentiation (Yeh et al. 1995). This is especially interesting because C/EBPβ is induced relatively early in adipogenesis (Cao et al. 1991) and can induce the expression of PPARγ (Wu et al. 1995). The differentiation stimulated by C/EBPβ appears to be mediated by PPARγ (Wu et al. 1995).

PPARγ, a member of the nuclear hormone receptor family, is selectively expressed in adipose tissue and is induced at an early stage of this differentiation process (Tontonoz et al. 1994b). PPARγ has been cloned and identified as a component of the adipogenic transcription factor ARF6 that bound to the enhancer of the adipocyte P2 (aP2) gene (Tontonoz et al. 1994a,b). Subsequently, it was demonstrated that ectopic expression of PPARγ converts several fibroblast cell lines into differentiation-competent preadipocytes (Tontonoz et al. 1994c). A number of diverse lipid and lipid-like compounds can activate PPARγ and stimulate adipogenesis in cells expressing this factor (Tontonoz et al. 1994b,c; Lehmann et al. 1995). Most recently, it has been demonstrated that thiazolidinediones, synthetic antidiabetic compounds, and 15–deoxy $\Delta^{12,14}$ prostaglandin (PG) J_2 are direct ligands of PPARγ and actively promote adipogenesis (Forman et al. 1995; Kliewer et al. 1995; Lehmann et al. 1995).

Though its function in adipogenesis is less understood than C/EBPα or PPARγ, a third transcription factor associated with adipogenesis is ADD1. ADD1 is a member of basic helix–loop–helix–leucine zipper (bHLH–LZ) family of transcription factors (Tontonoz et al. 1993). The basic domain of bHLH proteins binds to the target DNA recognition site termed E-boxes (CANNTG), and the HLH domain of these proteins controls homo- and/or heterodimerization (Murre et al. 1989; Benezra et al. 1990; Davis et al. 1990; Voronova and Baltimore 1990). That the bHLH proteins participate in development was first appreciated in muscle differentiation, but these factors have since been implicated in several developing cell types and lineages (Davis et al. 1987; Villares and Cabrera 1987; Caudy et al. 1988; Tapscott et al. 1988; Braun et al. 1989; Wright et al. 1989).

The discovery of developmentally regulated bHLH proteins in cell types such as skeletal myoblasts and neurons suggested that analogous bHLH proteins might exist in adipocytes. ADD1 is expressed at high levels in white fat, brown fat, and liver. Its mRNA expression is elevated in determined preadipocytes and increased further during the process of adipocyte differentiation (Tontonoz et al. 1993). The human homolog of ADD1 has been independently cloned as sterol regulatory element binding protein 1 (SREBP-1), a protein involved in the regulation of genes related to cholesterol metabolism (Yokoyama et al. 1993). Unlike other bHLH transcription factors, ADD1 has dual DNA-binding specificity, interacting with high affinity to both an E-box motif (ABS: atCACGTGta) and non-E-box sequence (ATCACCCCAC) (Kim et al. 1995), identified previously as sterol regulatory element 1(SRE–1; Smith et al. 1990). This unique dual DNA-binding specificity of the ADD1 homodimer is controlled by a single tyrosine residue in the basic domain (Kim et al. 1995). Several genes including fatty acid synthase (FAS) and S_{14} have been identified as containing a consensus ADD1-binding sequence (ABS) in their promoters, and these promoters can be *trans*-activated by the expression of ADD1 (Tontonoz et al. 1993; Kim et al. 1995). The tissue specificity of ADD1 and its ability to activate certain promoters suggest that ADD1 may play a significant role in adipocyte differentiation and lipogenesis.

In this study we investigate directly the role of ADD1 in adipogenesis. Ectopic expression of a dominant-negative ADD1 in 3T3–L1 preadipocyte cell line strongly inhibits the adipocyte differentiation and suppresses the expression of adipocyte-specific genes. We also examined the ability of ADD1 to induce adipocyte differentiation in nonadipogenic fibroblasts. In NIH–3T3 fibroblasts, overexpression of ADD1 and the application of differentiation-permissive culture conditions can induce increased morphological differentiation and the expression of adipocyte-specific genes. These data suggest that ADD1 is an important transcription factor in the process of adipocyte differentiation and fatty acid metabolism.

Results

Regulation of ADD1 expression during adipocyte differentiation

It has been previously demonstrated that ADD1 is expressed at high levels in liver and adipose tissue, including both white and brown fat. ADD1 mRNA was found to be elevated in determined preadipocytes, relative to other fibroblasts, and further elevated with overt differentiation (Tontonoz et al. 1993). Figure 1 illustrates the time course of ADD1 mRNA expression during adipocyte differentiation in both 3T3–F442A and 3T3–L1 cells. 3T3–F442A cells differentiate spontaneously at confluence in the presence of insulin and fetal calf serum, whereas differentiation of 3T3–L1 cells requires hormonal induction including dexamethasone (Dex), methylisobutylxanthine (MIX), insulin (Ins), and serum (see Materials and methods). Low levels of ADD1 mRNA are present in these preadipocytes at the time of cell confluence (day 0), and its expression is induced very early (1 day after confluence) in the differentiation of both cell lines (Fig. 1A,B). This increase is similar to or slightly precedes the induction of PPARγ and lipoprotein lipase (LPL) mRNA, two very early markers of adipocyte differentiation. Most adipocyte-specific mRNAs, including aP2, adipsin, and FAS, are detected between 3 and 5 days after confluence (Fig. 1). mRNA for C/EBPα, a tran-

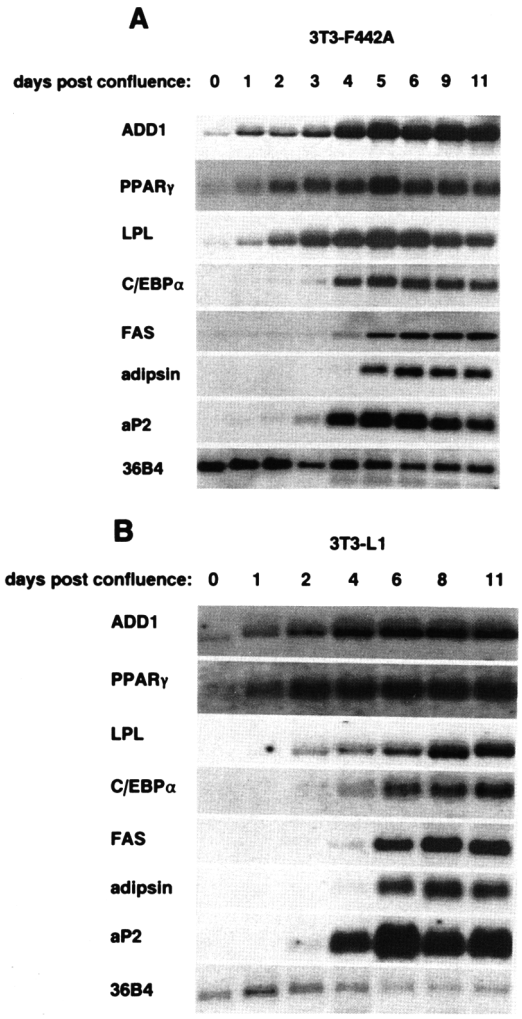

Figure 1. Induction of ADD1 mRNA during the differentiation of 3T3–F442A (*A*) and 3T3–L1 (*B*) preadipocytes. Total RNA (10 μg per lane) was isolated at the indicated time point after confluence, electrophoresed, blotted to nylon membrane, and hybridized with ^{32}P-labeled cDNA probes. Equivalent amounts of loaded RNA were run in each lane as indicated by hybridization to 36B4 cDNA. (FAS) Fatty acid synthase; (LPL) lipoprotein lipase.

scriptional regulator of many fat cell genes, is induced in the same period (3–4 days after confluence). This early induction of ADD1 mRNA in adipogenesis is consistent with the possibility that it could play a regulatory role in the adipocyte differentiation process per se or in the expression of certain genes expressed later in differentiation.

Construction of a dominant negative form of ADD1

We have demonstrated previously that ADD1 binds to specific DNA target sequences as a homodimer (Kim et al. 1995). The requirement for the formation of homo- or heterodimers is a common feature of the bHLH–LZ transcription factors. To test the biological role of ADD1, we sought to create a dominant-negative version. Because the basic region of bHLH factors is necessary for DNA binding but not dimerization, we constructed a dominant-negative allele of ADD1 (ADD1–DN), that contains an alanine residue at position 320 in the basic domain of a carboxy-terminal truncated, active form of ADD1 (amino acids 1–403). All other bHLH proteins except ADD1 contain a conserved arginine residue at this position, whereas wild-type ADD1 uniquely encodes a tyrosine residue here. It has been demonstrated that this tyrosine 320 is the key residue giving ADD1 its dual DNA-binding specificity (Kim et al. 1995). In an analysis of SREBP1 structure and function, it was demonstrated that a carboxy-terminal deletion of SREBP1 had higher DNA-binding activity and transcriptional activation compared to the full-length molecule (Sato et al. 1994). We have observed similar results and identified the superactive ADD1 (ADD1–403), which has the highest DNA-binding ability and transcriptional activity observed among several ADD1 deletion mutations. We put ADD1–403 and ADD1–DN into an expression vector, along with the full-length ADD1 molecule (ADD1) for DNA binding and transcriptional activation assays (see Materials and methods).

To examine the ability of ADD1–DN to bind DNA and to affect the DNA binding of other ADD1 molecules, ADD1–DN and a truncated, active form of ADD1–403 were produced by in vitro transcription and translation (see Materials and methods). We fixed the amount of the active form of ADD1–403 and mixed it with different amounts of ADD1–DN before adding the radiolabeled DNA probe in an electrophorectic mobility shift assay (EMSA). For DNA probe, an oligonucleotide containing the ADD1 consensus E-box target sequence was used (Kim et al. 1995). ADD1–DN, containing the alanine substitution at position 320, has completely lost the ability to bind to this DNA probe (Fig. 2A, lane 2). Also, addition of this protein to the active form of ADD1 (ADD1–403) interferes with its DNA binding in a dose-dependent manner.

The transcriptional activity of wild-type ADD1 has also been examined when cotransfected with vectors expressing ADD1–DN. ADD1–DN alone has no ability to activate transcription through the target sequence, presumably because of its inability to bind to DNA. As shown in Figure 2B, ADD1–DN dramatically inhibits the transcriptional activation stimulated by wild-type ADD1. This inhibition is almost complete at ratios of ADD1–DN to ADD1 plasmids of greater than 2:1.

Effects of ectopically expressed ADD1 alleles on adipocyte differentiation

To examine the role of ADD1 in adipocyte differentiation, we introduced this dominant-negative form of ADD1 (ADD1–DN) into 3T3–L1 preadipocytes via retrovirus infection. We have constructed several retroviral vectors with the superactive ADD1 (ADD1–403), ADD1–DN, and the full-length ADD1 (ADD1) cDNAs (Fig. 3A). For studies of adipogenesis, retrovirus systems have several advantages compared to the isolation of in-

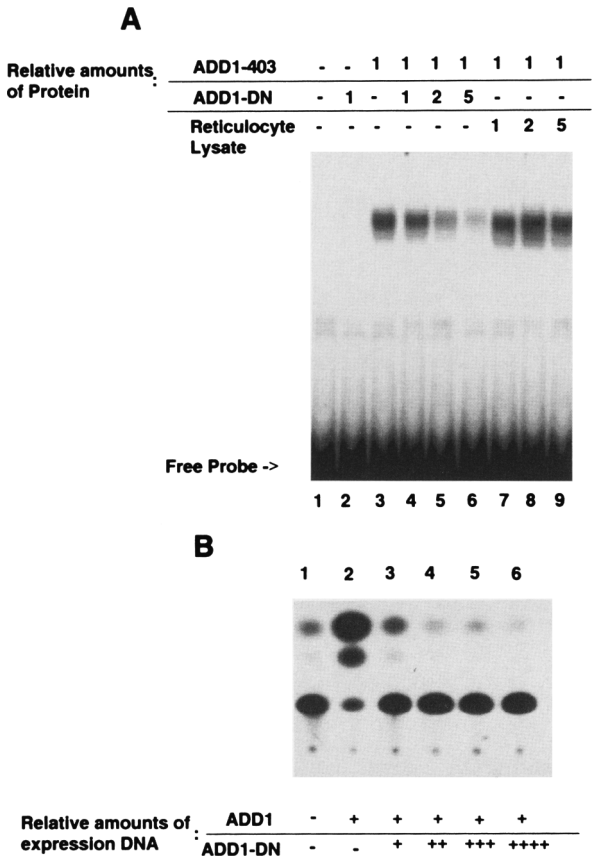

Figure 2. Functional analysis of a dominant-negative form of ADD1 (ADD1–DN). (*A*) Inhibition of DNA-binding activity of ADD1 by ADD1–DN. EMSA was performed with in vitro-translated ADD1–403 and ADD1–DN. During the reaction, different amounts of ADD1–DN and control reticulocyte lysate (lanes *4* and *7*, 1 μl; lanes *5* and *8*, 2 μl; lanes *6* and *9*, 5 μl of in vitro-translated ADD1–DN and reticulcyte lysate, respectively) were mixed with 1 μl of in vitro-translated ADD1–403 protein. Protein mixtures were then incubated at 37°C for 15 min to allow heterodimerization before the addition of double-stranded, ^{32}P-labeled, ABS probe (see the ABS sequence in Materials and methods). (*B*) Inhibition of transcriptional activation of ADD1 by addition of ADD1–DN. NIH–3T3 cells were cotransfected with the ABS CAT construct, 2 μg of ADD1 expression vector, and/or different amounts of ADD1–DN expression vector (lane *3*, 2 μg; lane *4*, 4 μg; lane *5*, 5 μg; lane *6*, 10 μg). The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity.

dividual cell clones transfected with DNA plasmids. First, the retroviral systems allow for the production of large homogeneous population of stably infected cells expressing a single or few copies of the introduced gene. Second, it minimizes clonal variation that can be quite severe in the study of adipogenesis (Green and Kehinde 1976). Several different ADD1 constructs were inserted into pBabe retroviral vector containing the Moloney murine leukemia virus long terminal repeat (MMTV LTR) (Fig. 3A). This vector also allows for expression of the selective marker gene for puromycin and ADD1. The parental pBabe or recombinant ADD1 retroviral vector was transfected into high efficiency viral packaging cell line BOSC23, and the resulting supernatant was used to infect cells (Pear et al. 1993).

Comparison of the growth rates of control 3T3–L1 preadipocytes infected with pBabe vector and of preadipocyte cell lines infected with ADD1–403, ADD1–DN, or ADD1 revealed no significant differences (data not shown). Northern blot analysis was performed to examine the mRNA expression levels of virally introduced ADD1 from each 3T3–L1-derived cell line. The 3T3–L1–ADD1–403, 3T3–L1–ADD1–DN, and 3T3–L1–ADD1 cell lines all express about twofold more of the exogenous ADD1 mRNAs compared to the amount of ADD1 mRNA observed in fully differentiated adipocyte cells (Fig. 3B). Both 3T3–L1–ADD1–403 and 3T3–L1–ADD1–DN cell lines produce a 4.2-kb fusion mRNA containing ADD1 cDNA encoding amino acids 1–403 and a viral mRNA. The size of exogenous mRNA from 3T3–L1–ADD1–403 or 3T3–L1–ADD1–DN is very close to the size of the endogenous ADD1 mRNA (Fig. 3B). In contrast, the 3T3–L1–ADD1 cell line expresses an mRNA of almost 6 kb because the ectopic transcript encodes the full-length ADD1 cDNA.

These 3T3–L1-derived cell lines were cultured to confluence and then treated with a standard differentiation-induction medium containing Dex, MIX, and Ins (see Materials and methods). After treatment with this medium for 2 days, the control 3T3–L1–vector cell line began to exhibit the accumulation of fat droplets and the rounded morphology typical of differentiated 3T3–L1 adipocytes by the third day after withdrawal of the induction medium. The cell lines containing the active forms of ADD1, 3T3–L1–ADD1-403 and 3T3–L1–ADD1, started to develop fat droplets by day 1 or 2 after induction (data not shown). After 5 or 6 days, 3T3–L1–ADD1–403 and 3T3–L1–ADD1 cell lines exhibit somewhat more lipid accumulation than the control 3T3–L1–vector cell line (Fig. 4 A,B). In contrast, the 3T3–L1–ADD1–DN cell line reveals minimal accumulation of cytoplasmic fat droplets and very little morphological change in the same time frame. Sporadic differentiation was visible only at day 5 or 6 in the 3T3–L1–ADD1–DN cells. This lower level of adipocyte conversion of the 3T3–L1–ADD1–DN cell line was observed even when the cells were maintained for 2 weeks after the induction of differentiation. These results were observed four times with two independently derived cell lines.

To characterize the differentiation of these cell lines at a molecular level, Northern blot analysis was performed to examine the expression of fat cell-specific genes. Total RNA was isolated at confluence and at day 4 after removal of the induction cocktail (day 6 after confluence). At day 0, no fat-specific gene expression is observed in any cell lines, except a small increase in LPL expression seen with the two active alleles of ADD1 (Fig. 5). As expected, expression of adipocyte-specific mRNAs encoding aP2, adipsin, PPARγ, C/EBPα, and LPL were greatly induced in the 3T3–L1–vector adipocytes with 6 days of incubation in differentiation medium (Fig. 5, lane

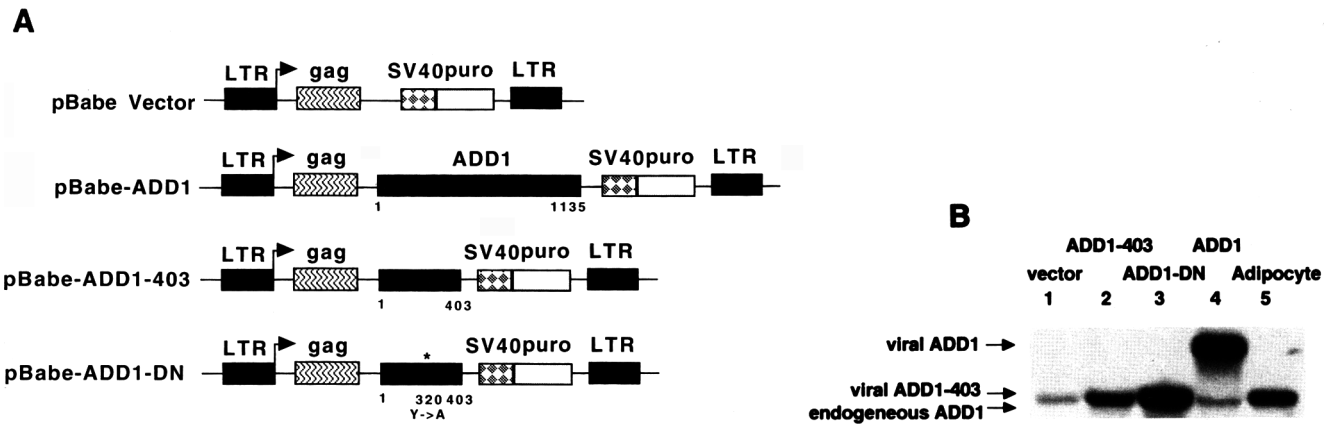

Figure 3. Construction of ADD1 retroviral vectors and ectopic expression in 3T3–L1 cell. (*A*) Schematic diagram of pBabe-derived retroviral vectors. MMTV LTR, gag, ADD1 cDNAs, and puromycin resistance genes are indicated. The arrows indicate the transcriptional initiation site. (*B*) Expression of retroviral ADD1 in 3T3–L1 cells. 3T3–L1 cells were infected with retrovirus carrying pBabe-derived expression vectors as described in Materials and methods. Total RNA (10 μg per lane) was isolated from virally introduced 3T3–L1 cell lines and differentiated 3T3–F442A adipocytes, electrophoresed, blotted to nylon membrane, and hybridized with ^{32}P-labeled ADD1 cDNA. Positions of the endogenous and exogenous ADD1 mRNAs are indicated.

5). In cell lines expressing wild-type ADD1 or ADD1–403, somewhat enhanced expression of most of these genes was observed relative to that seen in the 3T3–L1–vector cell line (Fig. 5, lanes 6,8). Consistent with the morphological observations, the suppression of most fat cell-specific gene expression was observed in the 3T3–L1–ADD1–DN cell line (Fig. 5, lane 7). These data suggest that ADD1 is required for execution of the adipocyte differentiation program at a morphological and molecular level.

Ectopic expression of ADD1 promotes adipocyte differentiation in NIH–3T3 fibroblasts

To examine the ability of ADD1 to promote adipogenesis in NIH–3T3 cells, a relatively nonadipogenic line, NIH–3T3 cells were infected with the same viral vectors including ADD1–403, ADD1, and the control pBabe vector and selected with puromycin. All cell lines grew at similar rates (data not shown). Immediately after confluence, these cells were cultured further in the absence or

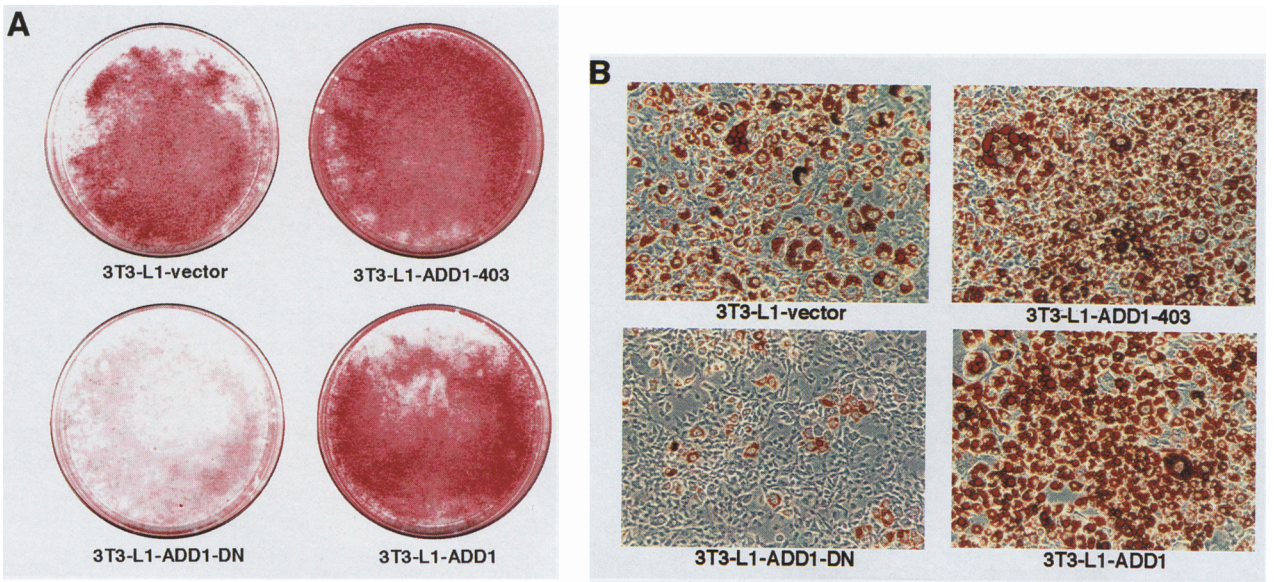

Figure 4. Effects of ectopic expression of ADD1 and ADD1–DN on 3T3–L1 preadipocyte differentiation. 3T3–L1–vector, 3T3–L1–ADD1–403, 3T3–L1–ADD1–DN, and 3T3–L1–ADD1 cell lines were treated with differentiation media (Dex, MIX, and Ins) for 48 hr at confluent culture and subsequently cultured for 6 days. Cells were fixed and stained with Oil Red O as described in Materials and methods. (*A*) Macroscopic view of total dishes; (*B*) microscopic views of dishes shown in *A* at 50× original magnification.

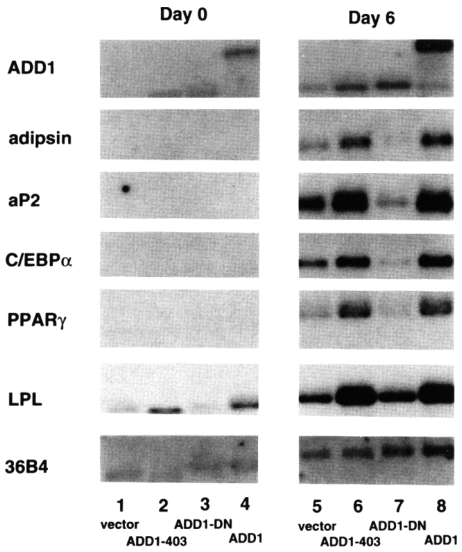

Figure 5. Transcriptional effects of expression of ADD1 and ADD1–DN on 3T3–L1 preadipocytes. Total RNA (10 μg per lane) was isolated at confluence (day 0) and after 6 days of confluent culture after treatment with induction media containing Dex, MIX, and Ins (day 6). Isolated RNA was separated by electrophoresis, blotted to nylon membrane, and hybridized with the indicated ^{32}P-labeled cDNAs. (Lanes *1,5*) RNA from 3T3–L1–vector; (lanes *2,6*) RNA from 3T3–L1–ADD1–403; (lanes *3,7*) RNA from 3T3–L1–ADD1–DN; (lanes *4,8*) RNA from 3T3–L1–ADD1.

presence of differentiation induction medium. To induce adipocyte differentiation in the NIH–3T3-derived fibroblasts, we tested several different adipogenic inducers. In 10% fetal calf serum (FCS) but without any induction cocktail, NIH–vector, NIH–ADD1–403, and NIH–ADD1 cell lines did not differentiate into adipocytes, indicating that ADD1 is not sufficient to promote differentiation under conditions that are not favorable for adipogenesis (data not shown). When these same NIH cell lines were exposed to a differentiation medium similar to that used for 3T3–L1 cells (Dex, MIX, and Ins; see Materials and methods), a small number (<3%) of the NIH–ADD1–403, and NIH–ADD1 cells phenotypically differentiated into adipocyte containing cytoplasmic lipid droplets, but no adipocyte differentiation was observed in NIH–vector cells (data not shown). Because it has been demonstrated recently that PPARγ is an important component in adipocyte differentiation, we repeated this analysis including a PPAR activator, 5,8,11,14-eicosatetraynoic acid (ETYA), in the differentiation medium (Dex, MIX, and Ins). When the cells were treated with this relatively strong differentiation-permissive media (10% FCS containing Dex, MIX, Ins, and ETYA; see Materials and methods) for 2 days, the NIH–vector control cells show a very small amount of differentiation (<3%; Fig. 6B). In contrast, the NIH–ADD1–403 and NIH–ADD1 cells showed much greater differentiation with ~15% and 25% of the cells showing the typical adipocyte morphology (Fig. 6A,B). A similar increase in adipogenesis in cells expressing ADD1 was observed in five independent experiments. These results suggest that ectopic expression of ADD1 in NIH–3T3 fibroblast may not be sufficient for the initiation of adipocyte differentiation under typical culture conditions but is able to promote the adipogenic program under strongly permissive conditions.

Total RNA was isolated from each of these cell lines at confluence and after 12 days of postconfluent culture with strong induction media. None of the NIH-derived

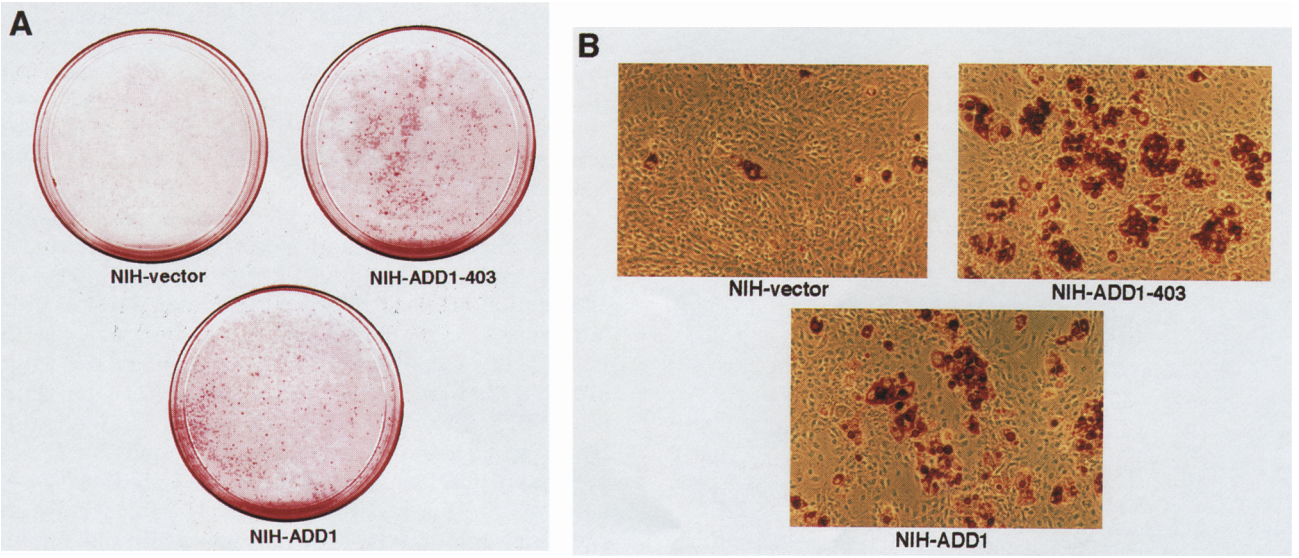

Figure 6. Ectopic expression of ADD1 in NIH–3T3 cells stimulates adipocyte differentiation. NIH–vector, NIH–ADD1–403, and NIH–ADD1 cell lines were treated with differentiation-permissive medium (Dex, MIX, Ins, and ETYA) and cultured for 12 days (see Materials and methods). Cells were fixed and stained with Oil Red O as described in Materials and methods. (*A*) Macroscopic view of total dishes; (*B*) microscopic views of dishes shown in *A* at 50× original magnification.

cell lines developed into visible fat cells at the time of cell confluence (day 0), and they do not induce the expression of aP2, adipsin, or PPARγ mRNA (Fig. 7, lanes 1–3). However, the NIH–ADD1–403 and NIH–ADD1 cell lines showed distinctively elevated expression of mRNA for LPL and FAS, key genes of fatty acid metabolism (Fig. 7). After 12 days of further culture, NIH–ADD1–403 and NIH–ADD1 cells activated the expression of many genes linked to adipogenesis, including aP2, adipsin, LPL, and PPARγ (Fig. 7, lanes 5,6). The induced expression of these adipocyte-specific genes closely correlates with the morphological change seen in the NIH–ADD1–403 and NIH–ADD1 cells. These data suggest that ectopic expression of active alleles of ADD1 in NIH–3T3 cell can promote the induction of the adipogenic program when provided with strong differentiation-permissive condition. In addition, ADD1 can induce the expression of the lipogenic genes including FAS and LPL without any differentiation inducing cocktail or any obvious morphological differentiation.

Subcellular localization of ADD1 proteins

We observed effects of both wild-type and carboxy-terminal-truncated ADD1 in adipogenesis and gene expression. This is somewhat surprising in light of recent reports that SREBP1 is held in an endoplasmic reticular membrane until released by proteolysis that is suppressed by cholesterol (Sato et al. 1994; Wang et al. 1994). Because we neither added nor depleted cholesterol in these experiments, we have investigated the subcellular localization of the wild-type and truncated alleles of ADD1. NIH–3T3 cells were transfected with expression vectors and were investigated by standard immunofluorescence and laser confocal microscopy. As shown previously in SREBP1 (Wang et al. 1994), the truncated ADD1–403 protein was detected only in nucleus as observed with standard immunofluorescence and laser confocal microscopy (Fig. 8A,B). In the cells transfected with full-length ADD1, large amount of ADD1 protein appeared to be located in the cytoplasm, nuclear membrane, and nucleus. The localization of ADD1 protein in both the nucleus and cytoplasm was clearly confirmed by laser confocal immunofluorescence microscopy (Fig. 8B). These results indicate that a substantial amounts of ADD1 protein derived from the wild-type allele has a nuclear localization.

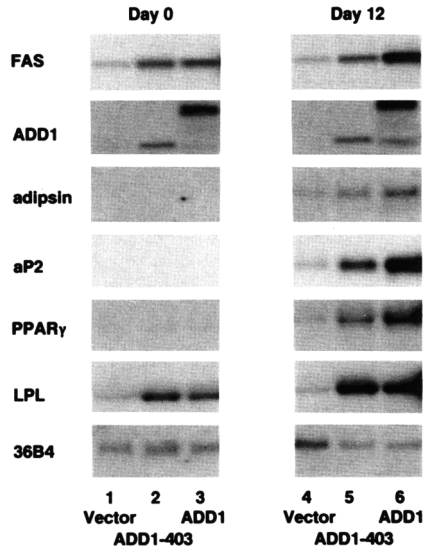

Figure 7. Expression of ADD1 in NIH–3T3 cells activates the transcription of adipocyte-specific genes. Total RNA (10 μg per lane) was isolated at confluence (day 0) and after 12 days of confluent culture under differentiation-permissive conditions (day 12). Isolated RNA was separated by electrophoresis, blotted to nylon membrane, and hybridized with the indicated ^{32}P-labeled cDNAs.

Expression of ADD1 enhances the transcriptional activation of PPARγ/RXRα

ADD1 could promote adipogenesis by activating a novel transcriptional pathway or by interacting with the known adipogenic regulators. Because both PPARγ and C/EBPα have been identified as key factors involved in adipocyte differentiation, we examined whether ADD1 affects the transcriptional activity of these two components. DNA target sites for PPARγ and C/EBPα are both found in the 5′-flanking region of the aP2 gene, and various reporter constructs are shown in Figure 9A. PPARγ binds to two sites in an upstream adipose-specific enhancer region as a heterodimer with RXRα (at approximately −5.2 kb), and C/EBPα binds to sites within the proximal promoter region (at −140 bp). Various combinations of ADD1, PPARγ, and/or C/EBPα were cotransfected with reporter genes containing binding sites for both C/EBPα and PPARγ [5.4-kb aP2 chloramphenicol acetyl transferase (CAT)], only PPARγ (520-bp aP2 CAT, ARE7 CAT), or only C/EBPα (168-bp aP2 CAT). The expression of ADD1 alone cannot induce the transcriptional activation of 5.4-kb aP2 CAT reporter, but cotransfection with PPARγ/RXRα enhances the transcriptional activity in the same CAT reporter (Fig. 9B). This cooperation activity between PPARγ/RXRα and ADD1 on the aP2 promoter appears to be by an indirect mechanism because ADD1 alone has no ability to activate transcription of the 5.4-kb aP2 CAT reporter. The cooperation between PPARγ/RXRα and ADD1 can also be observed in the 520-bp aP2 CAT construct (Fig. 9B, lanes 7–12). C/EBPα did not *trans*-activate the 5.4-kb aP2 CAT construct effectively, but the *trans*-activation of C/EBPα was observed in 168-bp aP2 CAT (Fig. 9B). However, cooperation between ADD1 and C/EBPα on 168-bp aP2 CAT reporter was not observed (Fig. 9B). Finally, we have asked whether ADD1 can increase the transcriptional activity of PPARγ on its minimal binding site. As shown in Figure 9B (lanes 19–24), ADD1 but not ADD1–DN stimulates PPARγ-induced transcriptional activity. These results indicate that ADD1 increases the transcriptional activity of PPARγ/RXRα heterodimer

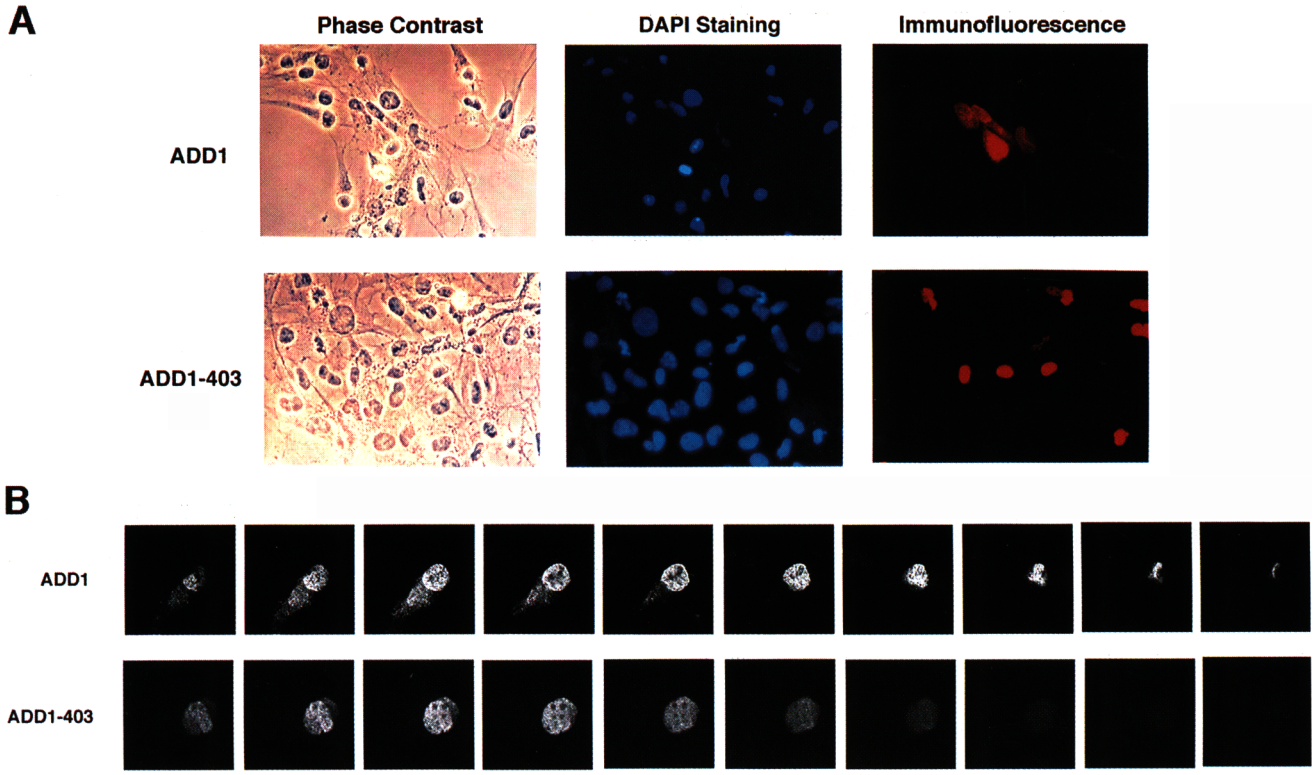

Figure 8. Subcellular localization of ADD1 protein. (*A*) Indirect immunofluorescence microscopy for full-length ADD1 and truncated ADD1–403. NIH–3T3 cells transfected with either ADD1 or ADD1–403 expression vectors were processed for immunofluorescence as described in Materials and methods and probed with anti-ADD1 polyclonal antibodies followed by rhodamine-conjugated anti-rabbit antibodies (*red*) in the *middle*. Coincident staining of the same cells with DAPI is shown on the *right* (*blue*), as well as phase contrast on the *left*. (*B*) Confocal laser scanning microscopy of the same cells as in *A*. Each picture was produced by serial Z-sectioning (0.4 μm).

and suggest that this may be one mechanism by which ADD1 promotes adipogenesis.

Discussion

ADD1 is unique among bHLH proteins in that it has a dual DNA-binding specificity (Kim et al. 1995), binding to both an E-box motif (Tontonoz et al. 1993) and non-E-box motif (SRE–1; Yokoyama et al. 1993). A role for ADD1 in activating the transcription of certain genes linked to adipocyte differentiation was suggested by its capacity to *trans*-activate the promoters of the FAS and S_{14} genes (Tontonoz et al. 1993; Kim et al. 1995). The human homolog of ADD1, SREBP1, is also involved in the regulation of several genes crucial for cholesterol homeostasis, and all of the known activities of SREBP1 in cholesterol metabolism is mediated by binding to a non-E-box motif (SRE-1) (Briggs et al. 1993; Hua et al. 1993; Wang et al. 1993; Yokoyama et al. 1993). With the dual DNA-binding specificity, we have suggested previously that ADD1/SREBP1 may be broadly involved in coordinating different pathways of lipid metabolism (Kim et al. 1995).

Although ADD1 is expressed abundantly in fat tissues, little was known concerning a role for this factor in adipocyte differentiation. In the present work we demonstrate that ADD1 plays an important role in this process. Several lines of evidence support this. First, a dominant-negative version of ADD1, which inhibits the DNA binding of the wild-type molecule, interferes with the process of differentiation in established preadipocyte cell line. Second, under conditions that are not permissive for a full differentiation, ADD1 expression activates the expression of at least two endogenous genes, LPL and FAS, linked to adipogenesis. Finally, though ADD1 expression is not sufficient to promote a great deal of differentiation under some differentiation-promoting conditions, it causes a large increase in adipogenesis when a PPARγ activator is included.

It is notable that ADD1 can induce two genes that are key regulators of fatty acid metabolism, LPL and FAS. Fat cells have the ability to synthesize triglycerides from fatty acids that are provided by circulating lipoproteins or via endogenous fatty acid biosynthesis. LPL is the key enzyme in the hydrolysis of blood-borne triglyceride-rich lipoproteins. LPL is synthesized and secreted from fat, and then binds to the luminal surface of the microvasculature. Under the influence of apolipoprotein CII, LPL hydrolyzes fatty acids from the triglyceride-rich core of very low density lipoprotein (VLDLs) and chylomicrons.

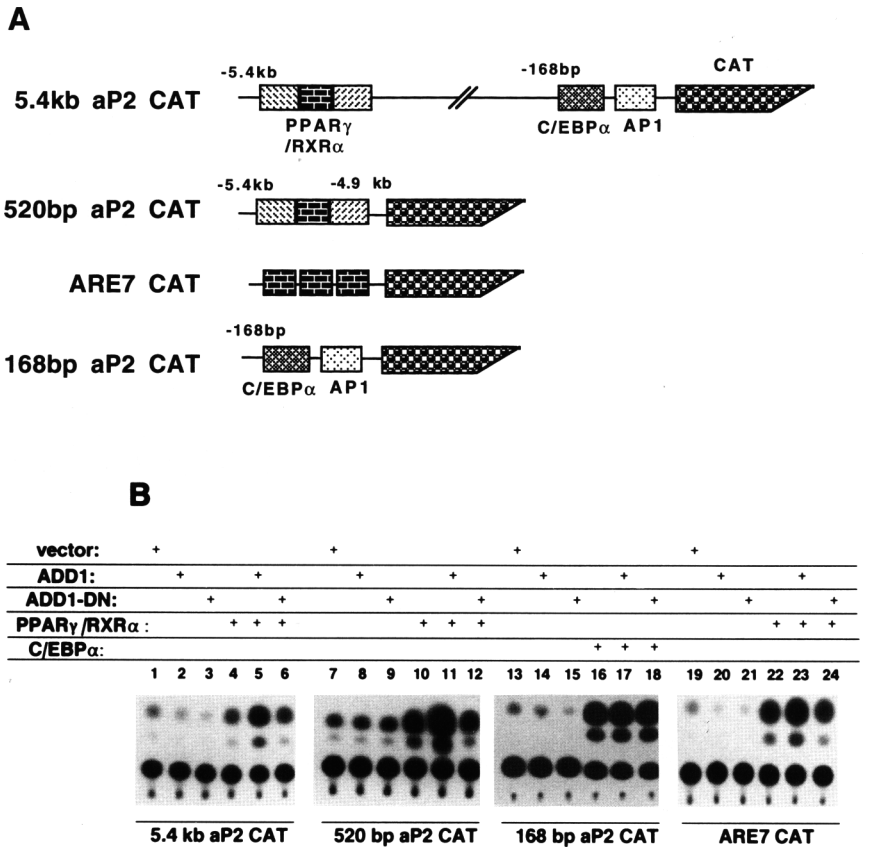

Figure 9. Enhancement of transcriptional activation of PPARγ/RXRα by ADD1 (*A*) Schematic diagram of the 5.4-kb aP2 CAT, 520-bp aP2 CAT, 168-bp aP2 CAT, and ARE7 CAT constructs. The binding sites for PPARγ/RXRα, C/EBPα, and AP1 factors are indicated. (*B*) NIH–3T3 cells were cotransfected with either 5.4-kb aP2 CAT, 520-bp aP2 CAT, 168-bp aP2 CAT or ARE7 CAT and the indicated combination of ADD1, ADD1–DN, and/or PPARγ/RXRα expression vectors. The level of CAT gene expression resulting from each transfection was determined by measuring CAT enzyme activity.

These unesterified fatty acids are then imported by fat cells and serve mainly as precursors for fat synthesis. Fatty acid synthase (FAS) is a large enzyme that can synthesize fatty acids de novo from C2 acetyl coenzyme A subunits, to be biosynthesized further into triglycerides. These data suggest that ADD1 may play a role in modulating the fatty acid flux in fat cells, and perhaps also in liver, in addition to a role in adipogenesis and cholesterol metabolism. The fact that these genes can be regulated by ADD1 in cells that are not undergoing adipogenesis suggests that this action may be very direct, perhaps through binding to the promoters of LPL and FAS. It has been demonstrated that the promoter of FAS gene contains both E-box and non-E-box (SRE-1) motif, which are binding sites for ADD1, and that these sites are important for the regulation of the FAS gene (Tontonoz et al. 1993; Bennett et al. 1995).

Recently, it has been appreciated that adipogenesis involves at least two families of transcriptional regulators: PPARs and C/EBPs. Although only PPARγ is expressed in a highly adipose-selective fashion, overexpression of C/EBPα, C/EBPβ, or PPARγ can induce adipogenesis (Freytag et al. 1994; Lin and Lane 1994; Tontonoz et al. 1994c; Wu et al. 1995; Yeh et al. 1995). Many fat cell-specific genes have binding sites for both families of factors in their promoters. Recently, it has been observed that PPARγ is induced by C/EBPβ, and C/EBPβ-induced adipogenesis requires addition of a PPARγ activator (Wu et al. 1995). Given the observation demonstrated in this paper, it is important to know whether ADD1 represents a third "direct" pathway to adipogenesis or whether it augments the activity of PPARγ or C/EBPs. It would be necessary to know the most important targets of these factors to answer this question definitively, but the transcriptional activity of PPARγ and C/EBPα can be ascertained on test plasmids. As shown in Figure 9, cotransfection of ADD1 augments the transcriptional activity of PPARγ but has no discernible effects on the activity of C/EBPα. The effect of ADD1 in increasing the activity of PPARγ can be observed on the whole 5.4-kb flank of the aP2 promoter, on the isolated 520-bp "fat enhancer" from this gene, or on small, multimerized PPAR-binding sites. Hence, it is likely to be a general phenomenon.

The cause of the increased activity of PPARγ seen with ADD1 expression is not known, but there are two obvious possibilities. First, ADD1 may stimulate the production of an endogenous PPARγ ligand. Many PPARγ activators are fatty acid derivatives and an unusual prostaglandin 15-deoxy $\Delta^{12,14}$ PG J_2, is a direct ligand of this receptor (Forman et al. 1995; Kliewer et al. 1995). Because all fatty acids in fat cells must arise via the endogenous or exogenous pathways, the induction of LPL and FAS by ADD1 may be very important in stimulating endogenous ligand production. A second possibility is that ADD1 may increase the expression of another protein factor that is limiting in the PPARγ response pathway. It is now understood that most, if not all, enhancer binding proteins interact with "coactivators" that connect these upstream factors with the general transcriptional machinery (Cavailles et al. 1994;

Halachmi et al. 1994). Such candidate factors have been identified for several nuclear receptors, suggesting that they are general features of this family. If such components are for limiting PPARγ activity in fibroblastic cells, an increase in a coactivator through ADD1 expression could give the results described here.

It has been demonstrated that SREBP1 has a transmembrane insertion domain, and this factor is localized into endoplasmic reticulum until liberated by proteolysis (Sato et al. 1994; Wang et al. 1994). This proteolysis process is induced by the absence of cholesterol, whereby SREBP1 may then induce the enzymes of de novo cholesterol biosynthesis and the LDL receptor (Wang et al. 1994). In the studies shown here, ADD1 protein derived from the transfected wild-type gene has clear-cut biological effects in adipocyte differentiation and gene expression without any particular modulation of the cholesterol levels normally present in serum. Standard immunofluorescence and confocal microscopy indicate that a significant fraction of this ADD1 is localized to the nucleus, although there is also some in the cytoplasm and perinuclear membranes. The carboxy-terminal truncation mutation of ADD1–403 is observed almost exclusively in the nucleus as was shown previously (Sato et al. 1994; Wang et al. 1994). Taken together, these data suggest that mechanisms must exist that cause ADD1 to become localized to the nucleus, apart from cholesterol-mediated proteolysis. One possibility is that the wild-type protein itself can enter the nucleus or it can enter with the assistance of factors that mask the putative transmembrane domain. Alternatively (and more likely), there may be other mechanisms whereby the ADD1 protein is cleaved to release it from membranes. Because cholesterol is not known to play any particular role in adipogenesis or fatty acid metabolism, it seems likely that other hormones or peptide factors could control the localization of this protein, perhaps through proteolytic mechanisms analogous to those already described in cholesterol regulation. Further analysis of the cellular metabolism of ADD1 in adipogenesis will clearly be required to answer these questions.

In conclusion, the data presented here demonstrated that ADD1 plays an important role in several cell culture models of adipogenesis. As this same molecule has been suggested to play a key role in cholesterol homeostasis, it will be interesting to determine how these related but separate functions are coordinated in an in vivo context.

Materials and methods

Cell culture and induction of differentiation

The 3T3–F442A cells were cultured and differentiated into adipocytes as described previously (Green and Kehinde 1974). NIH–3T3 cells were maintained in growth medium containing Dulbecco's modified Eagle medium (DMEM) with 10% bovine calf serum (Hyclone). After puromycin selection (2 μg/ml), virally infected NIH–3T3 cell lines were cultured to confluence (considered day 0 of differentiation program) and induced to differentiate by treating cells with differentiation-permissive medium (DMEM containing 10% FCS, 1 μM Dex, 0.5 mM MIX, 5 μg/ml of Ins and 50 μM ETYA). The differentiation induction medium was then replaced, and cells were fed every other day with DMEM supplemented with 10% FCS and 5 μg/ml of insulin. 3T3–L1 preadipocytes were grown in DMEM containing 10% bovine calf serum. Differentiation of 3T3–L1 cells was induced as described previously (Lin and Lane 1992). At postconfluence, the medium was changed with DMEM supplemented with 10% FCS, 1 μM Dex, 0.5 mM MIX, and 5 μg/ml of Ins for 2 days. Thereafter the medium was replaced every other day with 5 μg/ml of Ins and 10% FCS. Cells were fixed by 10% formaldehyde in phosphate-buffered saline and stained with Oil Red O.

RNA isolation and Northern blot analysis

Total RNA was isolated from cultured cells by guanidine isothiocyanate extraction as described previously (Tontonoz et al. 1993). Ten micrograms of RNA was denatured in formamide and formaldehyde, and electrophoresed in formaldehyde-containing agarose gels. RNA was blotted to BioTrans nylon membrane (ICN), and membranes were cross-linked, hybridized, and washed as directed by the manufacturer. DNA probes were labeled with [α-^{32}P]dCTP (6000 Ci/mmole) by the random prime labeling kit (Boehringer Mannheim). To control for RNA loading, all blots were hybridized with a cDNA probe for human acidic ribosomal protein 36B4.

Transient transfection and CAT assay

NIH–3T3 cells were cultured as described above and transfected 1 day before confluence by the calcium phosphate method as described previously (Kim et al. 1995). Construction of the 5.4-kb aP2 CAT reporter, 520-bp aP2 CAT reporter, 168-bp aP2 CAT reporter, ARE7 CAT reporter, and ABS CAT reporter was described previously (Ross et al. 1990; Graves et al. 1992; Kim et al. 1995). For the study of dominant-negative ADD1 in CAT assays, different amounts of ADD1–DN expression vector (1, 2, and 5 μg) was cotransfected with the fixed amount of wild-type ADD1 expression vector (2 μg) and 2 μg of ABS CAT reporter DNA. The ADD1 expression vector (2 μg) was cotransfected with each CAT reporter plasmid (2 μg) and/or PPARγ/RXRα expression vector (1 μg/1 μg). The level of CAT gene expression was determined by measuring CAT enzyme activity. All transfection experiments were performed in duplicate, and at least three different DNA preparations of each plasmid were tested.

EMSA and plasmids

EMSAs were performed as described (Kim et al. 1995). For construction of ADD1–403 and ADD1–DN plasmids, carboxy-terminal truncated ADD1 cDNAs containing amino acid residues from 1 to 403 were generated by PCR and cloned into plasmid pSVSPORT1 (GIBCO/BRL). These constructs were transcribed and translated in vitro using the TNT SP6-coupled reticulocyte lysate system (Promega). For binding assays, reactions were performed in a 20 μl volume containing in vitro translation lysate (1–6 μl) and ^{32}P-labeled probe (0.1 pmole) with incubation at 37°C for 15 min. The DNA sequence of double-stranded oligonucleotide used is as follows: ABS, 5′-GATCCTGATCACGTGATCGAGGAG-3′. DNA–protein complexes were resolved on a 5% polyacrylamide gel. The gel was dried and exposed to film at −70°C. The ADD1–403, ADD1–DN, and ADD1 viral expression vectors were constructed by ligation of ADD1 cDNAs into the *Eco*RI site of pBabe vector (Pear et al. 1993). The cDNAs of ADD1–403 and ADD1–DN encode the amino acids residues from 1 to 403 of ADD1 protein and were cloned by PCR. All of

the mutations were verified by DNA sequencing. The ADD1–DN construct that encodes the alanine reside at position 320 was also engineered by PCR and cloned into pSVSPORT1 and pBabe.

Virally infected cell line production

To generate recombinant retroviruses, the high efficiency packaging cell line BOSC23 was transiently transfected with 15 μg of the various recombinant retroviral vectors (pBabe and its derivatives) using the calcium phosphate DNA transfection method (Pear et al. 1993). Viral supernatants were collected 48 hr after transient transfection of BOSC23 cells. Titers of retrovirus were 10^5–10^6 cfu/ml. In all retroviral infections, recipient cells (NIH–3T3 or 3T3–L1) were incubated with retrovirus for 5 hr in the presence of 4 μg/ml of polybrene. Then cells were subcultured (1:3) 2 days after infection in medium containing puromycin (2 μg/ml) for selection.

Immunofluorescence and laser confocal microscopy

Polyclonal antibodies against ADD1 were produced by immunizing rabbits with a bacterilly produced ADD1 protein (Kim et al. 1995). For indirect immunofluorescence and laser confocal microscopy, NIH–3T3 cells were grown on coverslips, transfected with ADD1 or ADD1–403 expression vectors as described above. Cells were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and washed with PBS. Primary ADD1 antibodies were applied (1:1000 dilution), and secondary rhodamine-conjugated anti-rabbit antibodies (Jackson Lab) were incubated. DNA was counterstained with DAPI (Molecular Probes) prior to the mounting. Cells were examined using a confocal laser scanning microscope LSM 410 (Zeiss) equipped with an external argon–krypton laser (488 and 568 nm) and an external water-cooled argon ion laser (365 nm). Optical sections were digitally recorded.

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