Delta-interacting Protein A, a New Inhibitory Partner of CCAAT/Enhancer-binding Protein β, Implicated in Adipocyte Differentiation*

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CCAAT/enhancer-binding protein β (C/EBPβ) is expressed early during the adipocyte differentiation program and plays an important role in this process. In an attempt to identify novel proteins that interact with C/EBPβ, we performed a yeast two-hybrid screen with a preadipocyte cDNA library and identified a new co-regulator, delta-interacting protein A (DIPA). DIPA mRNA is expressed during adipocyte differentiation of clonal cell lines. DIPA interacts with C/EBPβ and -δ proteins in intact cells and inhibits their transcriptional activity but not that of C/EBPα. Stable overexpression of DIPA in preadipocytes partially inhibits adipocyte differentiation, whereas its gene silencing enhances this process. DIPA and C/EBPβ co-localize in the nucleus, and overexpression of DIPA in preadipocytes results in a partial inhibition of the mitotic clonal expansion which is critical for differentiation. Thus, DIPA is a novel partner of C/EBPβ that down-regulates early events of adipogenesis.

The increased adipose tissue mass associated with obesity results from an increase in the number and size of adipocytes. Adipocytes are highly specialized cells that play a critical role in energy homeostasis. A major role of adipocytes is to store large amounts of triglycerides during periods of energy excess and to mobilize these depots during periods of nutritional deprivation. An insight in the molecular mechanisms underlying adipogenesis may lead to the development of strategies for reducing the prevalence of obesity. In vitro experiments using established cell lines and primary cultures that mimic adipocyte differentiation in vivo have provided important information on adipose tissue development (1). The 3T3-F442A (2), 3T3-L1 (3), and Ob1771 (4) cell lines are well characterized models for the study of adipocyte differentiation at the molecular level. After growth arrest, confluent cells undergo adipogenesis. An insight in the molecular mechanisms underlying adipogenesis may lead to the development of strategies for reducing the prevalence of obesity. In vitro experiments using established cell lines and primary cultures that mimic adipocyte differentiation in vivo have provided important information on adipose tissue development (1). The 3T3-F442A (2), 3T3-L1 (3), and Ob1771 (4) cell lines are well characterized models for the study of adipocyte differentiation at the molecular level. After growth arrest, confluent cells undergo adipocyte differentiation when maintained under appropriate culture conditions. Commitment and differentiation of adipocytes are governed by transcription factors that are under the control of the combinatorial effects of hormonal, cell-cell, and cell-matrix interactions (5–7). Differentiation is accompanied by dramatic increases in the expression of adipocyte fatty acid-binding protein aP21 and lipid-metabolizing enzymes and by changes in the expression of cytoskeletal and extracellular matrix proteins (1, 8). The adipocyte differentiation program is controlled by the sequential expression of various transcription factors, i.e. members of the CCAAT/ enhancer-binding protein (C/EBP) family, the peroxisome proliferator-activated receptor (PPAR) family, and the adipocyte determination and differentiation factor-1 (ADD1/SEBP1c) (9). These transcription factors interact with each other to execute adipocyte differentiation, including lipogenesis and adipocyte-specific gene expression, which are pivotal for metabolism in adipocytes. Although the late events of adipose conversion have been well documented, the molecular mechanisms regulating the early development of adipocytes remain poorly understood.

Expression of C/EBPβ and C/EBPδ occurs at a very early stage of adipocyte differentiation; overexpression of C/EBPβ and C/EBPδ promotes adipogenesis of fibroblasts (10, 11) and up-regulates expression of C/EBPα and PPARγ2. The generation by homologous recombination of mice lacking C/EBPβ and C/EBPδ has clearly established the essential role of these two C/EBP's for the differentiation of adipocytes both in vitro and in vivo (12).

The early expression of C/EBPβ and -δ in preadipocytes is mediated through activation of ERK (extracellular signal-regulated kinase)- and CREB (cAMP-response element-binding protein)/ATF-1-dependent pathways (13). Previous studies showed that C/EBPβ acquires DNA binding activity during the mitotic clonal expansion (MCE) of preadipocytes that follows induction of differentiation (14) and that MCE is dependent on C/EBPβ (15). It has also been shown that C/EBPβ interacts with different factors and that its activity is modulated by post-transcriptional modifications such as site-specific phosphorylation by mitogen-activated protein kinases (16) and calcium/calmodulin-dependent kinase (17). In addition, in vitro studies have identified several protein kinases A and C phosphorylation sites (18) and site-specific glycogen synthase kinase-3 phosphorylation (19). Another level of post-transcrip-

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1 The abbreviations used are: aP2, adipocyte fatty acid-binding protein; BrdUrd, 5-bromo-2'-deoxyuridine; C/ATF, CEBP-related activating transcription factor; C/EBP, CCAAT/ enhancer-binding protein; DIPA, delta-interacting protein A; MCE, mitotic clonal expansion; PPAR, peroxisome proliferator-activated receptor; TBP, TATA-binding protein; DAPI, 4′,6-diamidino-2-phenylindole; GPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; siDIPA, small interfering DIPA; RNAi, RNA-mediated interference.
tional regulation for C/EBPβ is provided by the expression of three C/EBPβ isoforms, LAP* (38 kDa), LAP (35 kDa), and LIP (20 kDa), through the use of alternative translational start sites in the C/EBPβ mRNA (20) or through proteolysis (21, 22). Recently, C/EBPβ activity has been reported to be modulated by sumoylation (23, 24) and by acetylation (25). The present study was conducted to identify C/EBPβ partners present in the early steps of adipose conversion in order to gain more insights into the molecular mechanisms involved in adipose tissue development. We have isolated delta-interacting protein A (DIPA) as a partner of C/EBPβ. Transient transfections show that DIPA inhibits the transcriptional activity of C/EBPβ and -δ but not C/EBPα. Moreover, overexpression of DIPA in preadipocytes partially inhibits adipocyte differentiation, whereas partial loss of its expression by small interfering RNA (siRNA) enhances this process. We propose that the inhibitory effect of DIPA relates to a down-regulation of C/EBPβ and -δ-dependent transcriptional activity accompanied by a partial inhibition of mitotic clonal expansion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal bovine serum, 200 units/ml penicillin, and 50 μg/ml streptomycin (standard medium). For adipocyte differentiation of Ob1771 and 3T3-F442A cell lines, cells were shifted to standard medium supplemented with 17 nM insulin and 2 nM triiodothyronine (differentiation medium). Two days post-confluent 3T3-L1 cells were stimulated to differentiate by the addition of a hormonal mixture (0.5 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylmethyl-xanthine, and 170 nM insulin) to the standard medium for 2 days and maintained thereafter in standard medium with only 170 nM insulin. Typically, by day 6 more than 80% of the cells were fully differentiated into adipocytes. For 5-bromo-2′-deoxyuridine (BrdU) labeling, cells were plated on coverslips. 18, 20, 22, and 24 h after induction of differentiation, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and day 2 coverslips were fixed and processed for the analysis of BrdU incorporation and C/EBPβ localization as described previously (15).

**Two-hybrid Screening**—A yeast two-hybrid library was generated using cDNA from reverse transcribed RNA from 2 days post-confluent Ob1771 cells in Hybrid-Zap Vector (Stratagene). A bait plasmid, containing cDNA from reverse transcribed RNA from 2 days post-confluent 3T3-L1 cells was constructed. Two-hybrid screening was performed according to the manufacturer’s instructions by using SYBR green PCR Master Mix. The expression of selected genes was in all cases normalized to the expression of TATA-binding protein (TBP). The oligonucleotides for each target of interest, designed using Primer Express software (PerkinElmer Life Sciences) were (forward and reverse) the following: DIPA, 5′-CTGATCGAGGAGTGAACG-3′, 5′-CGACTCCGAAAAGCAGCAG-3′; aP2, 5′-CTGCGGGTGAATTCGTCAAAGG-3′, 5′-CTGCGGGTGAATTCGTCAAAGG-3′; TRP, 5′-CTCTTCAACAGATGCTCTGAT-3′, 5′-ATGATGCTGCAGAAATCGCC-3′.

**Plasmids**—The murine PPARγ2 (615 to +67) and aP2 (−185 to +19) promoters were cloned from genomic DNA using oligonucleotide primers based on published sequence (29, 30) into pGL2 Basic vector (Promega). The retroviral construct containing FLAG-tagged DIPA cDNA was derived from pCDNA3-DIPA and cloned into pAVk-BiP2 retroviral vector. Full-length C/EBPβ, -δ and -δ were cloned into Myc tag containing pCMV-tag3B vector (Stratagene). The constructs were checked by DNA sequencing.

**Western Blotting**—Whole cell extracts, SDS-polyacrylamide gel electrophoresis, blotting, and enhanced chemiluminescence were performed as described previously (31). Primary antibodies were rabbit anti-mouse C/EBPβ (Santa Cruz Biotechnology), rabbit anti-mouse aP2 (Santa-Cruz), mouse anti-c-Myc (Roche Applied Science), mouse anti-FLAG (Eurodemed), and rabbit anti-TBP (Santa-Cruz). Secondary horseradish peroxidase-conjugated antibodies were purchased from Promega.

**In Vitro Transcription and Translation**—FLAG-tagged full-length DIPA and TSC22 and Myc-tagged full-length murine PPARγ and C/EBPβ expression vectors were transcribed and translated using the TnT reticulocyte lysate kit (Promega) in the presence of [35S]methionine.

**Co-immunoprecipitation**—NIH3T3 cells were transiently co-transfected by calcium phosphate co-precipitation with C/EBPβ or PPARγ and DIPA or TSC-22 expression vectors. Two days later, cells were washed twice in ice-cold phosphate-buffered saline containing 0.5 mM Na2VO4, scraped in ice-cold gentle lysis buffer (50 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 50 mM NaF, 10 mM β-glycero-phosphate, 0.5 mM Na2VO4, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture), sonicated, and then centrifuged at 10,000 × g at 4 °C for 20 min. Supernatants were recovered, and protein concentrations were determined. Antibodies (10 μg/ml) against the Myc tag were added to 1 mg of protein lysates in a final volume of 0.5 ml followed by incubation at 4 °C for 1 h. Protein G-Sepharose beads were mixed with the immunoprecipitates for 1 h at 4 °C. Immunoprecipitates were recovered by centrifugation at 2,500 × g and washed three times with ice-cold lysis buffer. Immunoprecipitated proteins were eluted in 100 mM glycin-HCl, pH 2, rapidly neutralized and subjected to SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). Membranes were probed with the indicated primary antibodies and visualized by enhanced chemiluminescence. The same procedure was performed using in vitro translated proteins, and the gels were fixed, dried, and exposed to phosphorimaging screens.

**Immunofluorescence Staining**—Differentiated cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and blocked in 0.2% gelatin for 30 min. Cells were then incubated with C/EBPβ antibodies followed by Alexa
Identification of a Novel Protein That Interacts with C/EBPβ by Yeast Two-hybrid Screening—To identify proteins that interact with C/EBPβ, we screened a yeast two-hybrid cDNA library derived from Ob1771 preadipocytes, using mouse C/EBPβ lacking activating domains as bait. A total of 1.5 × 10^6 transformants were screened, yielding 69 positive yeast colonies in the presence of 8 mM 3-aminotriazole. A series of positive clones are shown in Fig. 1A. Retransformation experiments confirmed that C/ATF, UBC-9, and an unknown protein (similar to human DIPA) specifically interact with C/EBPβ (Fig. 1B) but not with unrelated bait proteins, compared with a negative control (pLamin C), a set of weak interaction partners (pBD-MUT/pAD-MUT), and a set of strong interaction partners (pBD-WT/pAD-WT) as described by the manufacturer (Stratagene).

C/ATF has been shown previously to interact with C/EBPβ using a zipper-blot method (33) indicating the existence of functional cross-talk between the C/EBP and C/ATF families for the integration of hormonal stimuli at the transcriptional level. Ubc9 is the conjugating enzyme involved in sumoylation (small ubiquitin-like modifier), a post-translational modification of proteins that can affect target protein function by altering the subcellular localization and/or antagonizing other modifications (34, 35). Recently, it has been shown that an inhibitory domain is conserved in the C/EBP family that is a target for sumoylation, which controls the transcriptional activity, and that sumoylation of C/EBPβ-1 (LAP+) is likely to be important in the functional differences observed between C/EBPβ-1 and C/EBPβ-2 (LAP) (23, 24). Altogether, these observations demonstrate the efficiency of our screen for C/EBPβ partners.

Two plasmids encoded identical 441-nucleotide expressed sequence tags of Mus musculus Unknown (GenBank accession number NM_198616) similar to the human DIPA (NM_006848) (36). The DIPA sequence contains a basic region-leucine zipper (bZIP) DNA binding and dimerization domain, which shares similarities with the transcription factor FRA1 (36).

Interaction of C/EBPβ with DIPA—Co-immunoprecipitation assays were performed to examine the direct interaction between DIPA and C/EBPβ. In vitro translated FLAG-tagged DIPA or FLAG-tagged TSC-22 and Myc-tagged C/EBPβ or Myc-tagged PPARγ (50 μl each) were mixed. Immunoprecipitation using anti-Myc antibodies was performed, and immunoprecipitates were subjected to SDS-PAGE and autoradiography (Fig. 2A). These experiments revealed that the DIPA-translated protein interacts with C/EBPβ protein but not with PPARγ used as a negative control. The interaction with DIPA and C/EBPβ is specific, as TSC-22, a leucine zipper-containing transcription factor, was not immunoprecipitated (Fig. 2A). To confirm further interaction between DIPA with C/EBPβ protein and other members of this family, we performed co-immunoprecipitation using lysates from transient transfections of NIH3T3 cells followed by immunoblotting. Immunoprecipitates using anti-Myc antibodies from cells co-transfected with empty vector, as well as with FLAG-tagged DIPA or with Myc-tagged PPARγ vectors, did not contain any FLAG-tagged DIPA protein. However lysates from cells co-transfected with FLAG-tagged DIPA and either Myc-tagged C/EBPβ or δ -PPARγ vectors contained a clear band corresponding to the FLAG-tagged DIPA protein (Fig. 2B). There was no co-immunoprecipitation of DIPA when Myc-tagged C/EBPα was used (Fig. 2B). These findings indicate an interaction between DIPA and C/EBPβ and δ proteins in intact cells.

Indirect immunofluorescence microscopy was used to determine the subcellular localization of DIPA in 3T3-L1 cells that had been infected with pAkvi-FLAG-tagged DIPA. Nuclear staining of C/EBPβ protein was observed in cells (Fig. 2C) in agreement with reported data (37). A similar localization was observed for the FLAG-tagged DIPA (Fig. 2C). These patterns are consistent with a co-localization of C/EBPβ and DIPA in the nucleus as confirmed by the yellow staining of the merge micrograph (Fig. 2C).

Differential Effect of DIPA on the Transcriptional Activity of C/EBPα, -δ, and -β on the aP2 and PPARγ2 Promoters—The transcriptional activity of C/EBPα, -δ, and -β on the mouse aP2 and PPARγ2 promoters linked to a luciferase reporter gene was determined by transient transfections in NIH3T3 cells. The aP2 promoter (−185 to +19) and the PPARγ2 promoter (−615 to +67) have already been shown to contain C/EBP-responsive elements (38, 39). C/EBPα, -δ, and -β transactivated the aP2 promoter in a dose-dependent manner from 2- to 6-fold (Fig. 3). Transient transfections of DIPA alone inhibited the basal promoter activity by 30–50%, whereas it completely abolished the
activity of C/EBPβ, whatever the doses of DIPA expression vector (Fig. 3A), and moderately inhibited the activity of C/EBPβ in a dose-dependent manner (Fig. 3B). There was no effect of DIPA on the transcriptional activity of C/EBPα (Fig. 3C). As C/EBPβ is unable to activate PPARY2 promoter (40), we analyzed the effects of C/EBPα and -δ on the PPARY2 promoter in the absence or presence of the DIPA expression vector. The data show a negative effect of DIPA on C/EBPβ but not on C/EBPα transcriptional activity on the PPARY2 promoter (Fig. 4A and B).

Adipocyte Differentiation Is Modulated by DIPA Overexpression and DIPA Gene Silencing—As a first step, we examined the expression of DIPA mRNA as a function of differentiation of clonal 3T3-L1 and 3T3-F442A preadipocytes. As shown in Fig. 5, DIPA mRNA is expressed in growing cells and in the differentiating adipocytes. Consistent with this observation, DIPA mRNA was present in both stromal vascular and adipocyte fractions of white and brown adipose tissues (data not shown). Of note, the Northern blotting experiments indicate that the level of DIPA mRNA may decrease slightly at the start of the clonal expansion period (Fig. 5). However, the biological significance of this remains to be established and will require data on DIPA protein expression and possibly post-translational modification.

We next investigated whether DIPA overexpression in 3T3-L1 cells could affect their differentiation into adipocytes. Retroviral vectors were used to generate stable 3T3-L1 cell lines that overexpressed DIPA. The levels of exogenous DIPA mRNA are 3–4-fold higher than the endogenous mRNA (Fig. 6D, arrows I and 2). The extent of adipocyte differentiation of these stable cell lines was judged by Oil Red O staining of cellular lipids, GPDH activity, and the expression of adipocyte markers. Cells overexpressing DIPA exhibited lower levels of lipid accumulation compared with cells harboring the empty vector (Fig. 6A). Examination of the cultures by light microscopy revealed that the decrease in lipid staining in 3T3-L1-DIPA was due to a decrease in the total number of differentiated cells (Fig. 6A). 3T3-L1-DIPA cells expressed low levels of GPDH activity and aP2 protein compared with 3T3-L1-vector cells (Fig. 6, B and C). Further analysis at the molecular level showed that the C/EBPβ and -δ mRNA contents were similar both in stable cell lines and in nontransduced differentiating 3T3-L1 cells. In contrast, mRNA levels of target genes, i.e. C/EBPα, PPARY, and aP2 genes, were reduced in 3T3-L1-DIPA cells compared with control cells (Fig. 6D).

To further examine the role of DIPA in the adipocyte differentiation process, we reduced DIPA gene expression using an siRNA retrovirus vector. Two target sequences of the DIPA gene were selected for gene silencing and cloned in pSUPER retroviral vector. The effects of siRNAs were assayed by quantitative reverse transcription-PCR. DIPA gene expression levels were reduced by 40% using the siDIPA2 sequences (Fig. 7B), whereas siDIPA1 sequences were without effect. Reducing the level of DIPA gene expression by the siRNA retroviral vector resulted in an increase in the level of adipocyte differentiation as judged by morphological analysis and quantitative reverse transcription-PCR analysis of aP2 mRNA (Fig. 7). 3T3-L1 control cells and cells expressing a scrambled siRNA vector resulted in differentiation of ~80% of the cell population; however more than 95% of the cell population expressing the DIPA siRNA vector differentiated and accumulated lipid droplets. These data show that the levels of DIPA mRNA modulate adipogenesis, indicating that interaction with C/EBPβ and -δ could play an important role in this process.

Effect of DIPA Overexpression on Mitotic Clonal Expansion—It has been shown recently that MCE is required for adipogenesis and that C/EBPβ is crucial for this process (15, 37, 41). MCE precedes expression of the adipocyte genes that give rise to the adipocyte phenotype. We considered the possibility that DIPA and C/EBPβ interfere with MCE. To test this hypothesis, DNA synthesis was measured by BrdUrd labeling, 18, 20, 22, and 24 h after induction of differentiation, preadipocytes harboring the control or DIPA retroviral vectors were pulse-labeled for 2 h with BrdUrd. Forty-eight hours after induction of differentiation, cells were immunostained with anti-BrdUrd. As shown in Fig. 8A, 70% of 3T3-L1-vector cells exhibited BrdUrd incorporation into nuclear DNA when labeled between 20 and 22 h. In contrast, preadipocytes harboring the DIPA retroviral vector exhibited a lower proportion of cells (40%) incorporating BrdUrd. The same reduction in the proportion of
C/EBPα and DIPA Interaction Affects Adipogenesis

**FIG. 3.** DIPA inhibits the transcriptional activity of C/EBPβ and -δ, but not C/EBPα, on the aP2 minimal promoter. NIH3T3 cells were co-transfected in 24-well plates with expression vectors for C/EBPβ (A), C/EBPδ (B), and C/EBPα (C) and DIPA, using the indicated amounts, and with the luciferase reporter driven by the aP2 promoter (-185 to +19) containing a C/EBP-responsive element. PCMV-β-galactosidase vector was used as an internal control of transfection. Cells were lysed 48 h later, and then luciferase and β-galactosidase activities were measured. The numbers (RLU/β-gal, relative luciferase units/β-galactosidase activity) indicate the fold induction of the normalized luciferase activity compared with control. Data are given as mean ± S.D. of three independent experiments performed in triplicate. *, p < 0.01 versus corresponding control in the absence of added DIPA expression vector; †, not significant.

**FIG. 4.** DIPA inhibits C/EBPδ, but not C/EBPα, transcriptional activity on the PPARγ2 promoter. NIH3T3 cells were co-transfected and analyzed as described for Fig. 3 using a PPARγ2 promoter reporter and either C/EBPδ (A) or C/EBPα (B). Data are given as mean ± S.D. of three independent experiments in triplicates. *, p < 0.05; **, p < 0.01 versus corresponding control in the absence of added DIPA expression vector; †, not significant. RLU/β-gal, relative luciferase units/β-galactosidase activity.

**FIG. 5.** DIPA mRNA expression during adipocyte differentiation of 3T3-L1 and 3T3-F442A cells. Total RNA was extracted from preadipocyte cell lines 3T3-L1 and 3T3-F442A. Cells were maintained in differentiation media as described under “Experimental Procedures.” 20 µg of total RNA was loaded in each lane and analyzed by Northern blot. The results are representative of three independent experiments.

C/EBPβ is one of the key factors triggering adipogenesis. C/EBPβ is expressed early during differentiation, and its expression precedes that of C/EBPα, which is initiated concomitantly with growth arrest. Recent studies showed that C/EBPβ activity during the adipocyte differentiation process is affected by different signaling pathways. However, little is known about the involvement of activating and inhibiting partners. In an attempt to identify new co-regulators of C/EBPβ activity, we identified an inhibitory partner called DIPA. DIPA was cloned as a protein interacting with hepatitis delta antigen (HDAg), a protein that is required for viral replication. The interaction between DIPA and HDAg negatively modulates viral replication (36). DIPA mRNA is expressed in a human hepatic cell line and in most human tissues and encodes for a 24-kDa protein. The DIPA sequence is consistent with it being a DNA-binding protein, as it contains a basic region-leucine zipper DNA binding and dimerization domain, which has some similarities with transcription factor FRA1 and other FRA family members (36). Members of this family have been shown to play a role during adipocyte differentiation (42). Whether DIPA also functions as a regular transcription factor remains to be established. In this report we provide compelling evidence that DIPA interacts with C/EBPβ and that this interaction modulates the process of adipocyte differentiation. Our data show clearly that DIPA interacts with C/EBPβ and -δ but not with C/EBPα. This interaction leads to the inhibition of the transcriptional activity of C/EBPβ and -δ on the promoter of two genes specifically expressed during adipocyte differentiation, namely aP2 and PPARγ2 (Figs. 3 and 4). In our hands, C/EBPβ failed to activate the PPARγ2 promoter, in agreement with the report of Elberg et al. (40). Preliminary data show that DIPA does not affect C/EBPβ or -δ binding to AE1 (data not shown), a C/EBP-responsive element present in the aP2 promoter. The interaction of DIPA with C/EBPβ and -δ is specific, because a leucine zipper-containing transcription factor, TSC-22 (43), does not interact with C/EBPβ or -δ nor does DIPA interact with a zinc finger-containing transcription factor, PPARδ (Fig. 2B).

Retroviral overexpression of DIPA in 3T3-L1 preadipocytes partially inhibits the adipocyte differentiation process. 80% of the cell population expressing empty vector accumulates lipid droplets, whereas only around 45% of the cell population expressing DIPA accumulate lipids and exhibit reduced expres-
FIG. 6. Adipocyte differentiation of 3T3-L1 cells expressing FLAG-tagged DIPA. 3T3-L1-vector and 3T3-L1-DIPA cells were maintained after confluence in differentiation medium as described under "Experimental Procedures." A, cells were fixed and stained with Oil Red O at day 10 after induction of differentiation. B, GPDH activities were determined at the indicated times. C, Western blot analysis of the exogenous DIPA and aP2 expression with anti-FLAG and anti-aP2 antibodies was performed (50 μg of protein/lane). D, RNA was extracted on day 10 after induction of differentiation, and 20 μg was analyzed by Northern blot. Lanes a, b, and c are 3T3-L1, 3T3-L1-vector, and 3T3-L1-DIPA cells, respectively. Arrows 1 and 2 indicate exogenous and endogenous DIPA mRNA, respectively. The results are representative of two independent experiments.

FIG. 7. DIPA gene silencing enhances adipocyte differentiation. 3T3-L1-scramble and 3T3-L1-siDIPA2 cells were maintained after confluence in differentiation medium as described under "Experimental Procedures." A, cells were fixed, stained with Oil Red O, and photographed at day 10. B, RNA was isolated from 3T3-L1-scramble and 3T3-L1-siDIPA2 cells. The relative mRNA expression of DIPA and aP2 was measured using real-time PCR. TBP was used as an internal standard. RNA expression was normalized to TBP. Values are means ± S.D. from a representative experiment, *, p < 0.05. The experiments were performed in triplicate and repeated two times.

FIG. 8. Effect of DIPA overexpression on mitotic clonal expansion. Stable 3T3-L1-vector and 3T3-L1-DIPA cells, plated on coverslips, were induced to differentiate in differentiation medium and 20 h after induction were pulse-labeled for periods of 2 h with BrdUrd, as described under "Experimental Procedures." A, BrdUrd-labeled nuclei were determined as BrdUrd-positive nuclei divided by total nuclei (DAPI-stained). Data are given as mean ± S.D. of three independent experiments performed in duplicate; *, p < 0.05. B, incorporation of BrdUrd into the DNA was detected with an anti-BrdUrd (green), C/EBPβ (red), nuclei shown in blue are stained with DAPI. Scale bar, 20 μm.
sion of key markers such as GPDH, aP2, PPARγ, and lipoprotein lipase (LPL) (Fig. 6). DIPA gene silencing using siRNA retroviral vector enhances somewhat the proportion of differentiat-

eated cells allowing lipid droplets to accumulate in the whole cell population and a modest increase in the level of aP2 mRNA expression. Altogether, these observations show that the level of DIPA mRNA modulates the adipocyte differentiation process. This is consistent with preliminary results showing that drugs negatively affecting adipocyte differentiation, such as tumor necrosis factor-α, increase the level of DIPA mRNA (data not shown).

It is well established that expression of C/EBPβ occurs early in the differentiation program and is a prerequisite for MCE (15, 37). Overexpression of DIPA in 3T3-L1 preadipocytes affects this process by reducing the number of cells in the S-phase of the cell cycle (Fig. 8). The mechanism by which DIPA affects adipocyte differentiation is not known, although we can exclude an effect on the mRNA turnover of C/EBPβ and on the ratio of LIP versus LAP protein level (data not shown). As C/EBPβ and DIPA co-localize in the nucleus, it is tempting to postulate that C/EBPβ is sequestered by DIPA and that this sequestering prevents both its positive effects on mitotic clonal expansion and adipocyte differentiation. Post-translational modifications of C/EBPβ have been shown to modify its activity (16–19, 23–25). Such modifications brought by the hormonal environment and Dr. R. Arkowitz for helpful suggestions and crit-

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