Functional antagonism between inhibitor of DNA binding (Id) and adipocyte determination and differentiation factor 1/sterol regulatory element-binding protein-1c (ADD1/SREBP-1c) *trans*-factors for the regulation of fatty acid synthase promoter in adipocytes

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We show that Id (inhibitor of DNA binding) 2 and Id3, dominant negative members of the helix–loop–helix (HLH) family, interact with the adipocyte determination and differentiation factor 1 (ADD1)/sterol regulatory element-binding protein (SREBP) 1c, a transcription factor of the basic HLH-leucine zipper family that controls the expression of several key genes of adipose metabolism. Gel mobility-shift assays performed with *in vitro*-translated ADD1, Id2 or Id3 proteins and a fatty acid synthase (FAS) promoter oligonucleotide showed evidence for a marked inhibition of the formation of DNA–ADD1 complexes by Id2 or Id3 proteins. Co-immunoprecipitation studies using *in vitro*-translated proteins demonstrated further the physical interaction of Id and ADD1/SREBP-1c proteins in the absence of DNA. Using the *FAS* gene as a model of an ADD1-regulated promoter in transiently transfected isolated rat adipocytes or mature 3T3-L1 adipocytes, a potent inhibition of the activity of the FAS-chloramphenicol acetyltransferase reporter gene was observed

by overexpression of Id2 or Id3. Reciprocally, co-transfection of Id3 antisense and ADD1 expression vectors in preadipocytes potentiated the ADD1/SREBP-1c effect on the FAS promoter activity. Finally, in the non adipogenic NIH-3T3 cell line, most of the ADD1-mediated *trans*-activation of the FAS promoter was counteracted by co-transfection of Id2 or Id3 expression vectors. Previous studies have indicated *Id* gene expression to be down-regulated during adipogenesis [Moldes, Lasnier, Fève, Pairault and Djian (1997) *Mol. Cell. Biol.* 17, 1796–1804]. We here demonstrated that there was a dramatic rise of Id2 and Id3 mRNA levels when 3T3-L1 adipocytes or isolated rat fat cells were exposed to lipolytic and anti-lipogenic agents, forskolin and isoproterenol. Taken together, our data show that Id products are functionally involved in modulating ADD1/SREBP-1c transcriptional activity, and thus lipogenesis in adipocytes.

Key words: helix–loop–helix, lipogenesis, 3T3 adipocytes.

INTRODUCTION

Helix–loop–helix (HLH) transcription factors have been shown to regulate an increasing number of developmental processes in different organisms. Due to their ability to dimerize, these factors form an interacting network that regulates the activity of many genes implicated in cellular growth, determination and differentiation (reviewed in [1]).

Several subfamilies of HLH proteins can be distinguished by the presence (basic HLH, or bHLH) or the absence (dominant negative HLH, or dnHLH) of a DNA-binding basic region directly adjacent to the HLH domain [2,3]. The HLH dimerization domain consists of two amphipathic α -helices separated by a loop of amino acids. Homodimer and/or heterodimer formation is thought to be mediated by complementary residues of helix domain [4] while the basic domain is responsible for binding to DNA [5–7]. Members of the bHLH family bind variations on the core sequence CANNTG, referred to as the E-box motif [8]. Ids (inhibitors of DNA-binding), ubiquitously expressed, are dnHLH proteins. They are believed to act as negative regulators of DNA binding by sequestering bHLH factors [2,9–11]. Besides these two major subfamilies, there exists a group of *trans*-activators termed bHLH-LZ which contains an

additional leucine-zipper domain immediately C-terminal to the bHLH domain.

During the work on adipose conversion, a nuclear factor of the bHLH-LZ family, termed adipocyte determination and differentiation factor 1 (ADD1), was cloned from rat adipose tissue [12]. It belongs to the sterol regulatory element-binding protein (SREBP) family, a class of *trans*-factors released from membranes by proteolysis. These factors were cloned independently and studied extensively in the field of the cholesterol-regulated gene expression by the group of Brown and Goldstein [13–15]. ADD1, also called SREBP-1c, regulates the expression of several key genes of fatty acid and triglyceride metabolism in cultured fibroblasts, adipocytes and liver of transgenic mice [16,17]. This factor, strongly induced during the adipose conversion, is a potent *trans*-activator of the fatty acid synthase (FAS) promoter. Molecular dissection of the FAS promoter has shown previously that ADD1/SREBP-1c acts through an E-box motif at nucleotides –64 to –59 [18], a sequence identified previously as the major insulin-response element of this gene ([19,20], reviewed in [21]).

So far, no partner controlling the transcriptional activity of ADD1/SREBP-1c has been identified in the adipocyte. A previous report from our laboratory has documented the pre-

Abbreviations used: Id, inhibitor of DNA binding; ADD1, adipocyte determination and differentiation factor 1; SREBP, sterol regulatory element-binding protein; HLH, helix–loop–helix; bHLH, basic HLH; dnHLH, dominant negative HLH; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco–Vogt modified Eagle's medium; FAS, fatty acid synthase; USF, upstream stimulatory factor; bHLH-LZ, bHLH-leucine zipper; RSV, Rous sarcoma virus.

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ventative role of Id3 in the adipose-conversion process [22]. Given the structural relationship between ADD1/SREBP-1c and Id proteins, the important role of ADD1/SREBP-1c in the regulation of fat metabolism and adipocyte development, and the negative effect of Id3 on adipose differentiation, we investigated the possible interaction between these factors for controlling FAS-promoter activity. Here we demonstrate that Id and ADD1/SREBP-1c proteins can heterodimerize *in vitro*. Using transient co-transfection experiments, we provide evidence for functional antagonism between Id2 or Id3 and ADD1/SREBP-1c proteins in adipocytes and NIH-3T3 fibroblasts. Whereas Id mRNA levels are strongly down-regulated when cells enter into terminal differentiation, we observe a reversal of Id gene expression after adipocyte exposure to effectors of the cAMP signalling pathway. In this context, the functional interaction described in this study is likely to be of physiological relevance in the modulation of lipogenesis.

MATERIALS AND METHODS

Plasmid constructs

The backbone pcDNA3 (Invitrogen) contained a near full-length Id3 cDNA (positions 18–924 bp of cDNA sequence, described in [9]) in the sense or antisense orientation [22]. These cDNAs were cloned into an *EcoRI* site of the polylinker immediately downstream of the CMV (cytomegalovirus) promoter. The Id2 full-length cDNA (a gift from Dr T. Neuman, Fort Collins, CO, U.S.A.) [23] was cloned into the pRc-CMV vector (Invitrogen) between *HindIII/XbaI* sites in the sense orientation. Since pcDNA3 and pRc-CMV vectors only differ in their polylinker, the pcDNA3 vector was used as the control empty vector in transient-transfection experiments.

The ADD1 expression vector encoding the first 403 amino acids of rat ADD1, and the plasmid ADD1-DN encoding a dominant negative form of ADD1 unable to bind DNA [16], were cloned in pSVSPORT1 plasmid and were gifts from Dr. B. M. Spiegelman (Dana-Farber Cancer Institute, Boston, MA, U.S.A.). The full-length cDNA encoding the 43-kDa human upstream stimulatory factor (USF) [24] was subcloned in the pBS-KS+ vector (Stratagene).

The FAS-chloramphenicol acetyltransferase (CAT) plasmid containing a –118/+65 fragment of FAS promoter in front of a CAT reporter gene was described previously [25]. In some experiments, a mutated version of the FAS-CAT plasmid in which the E-box CATGTG has been turned to CTGTAC was used as a negative control. Site-directed mutagenesis was performed using the Quick Change kit (Stratagene) according to the manufacturer's instructions. The internal control pRSV- β -gal (where RSV is Rous sarcoma virus) plasmid has been described elsewhere [26]. Plasmids were purified using the CsCl centrifugation method or a commercial kit (Nucleobond AX; Macherey-Nagel, Hoerd, France).

Cell culture and preparation

Murine 3T3-L1 preadipose cells [27] were grown until confluence in Dulbecco–Vogt modified Eagle's medium (DMEM; 4.5 g of glucose/l) supplemented with 10% fetal calf serum. At the time of confluence (day 0), differentiation of 3T3-L1 cells was induced with methyl-isobutylxanthine (100 μ M), dexamethasone (0.25 μ M) and insulin (1 μ g/ml) [28] for 48 h and the cells then were re-fed with DMEM containing 10% fetal calf serum and insulin (1 μ g/ml). Culture medium was changed every other day. More than 90% of the cells acquired an adipocyte morphology 8 days after the initiation of differentiation. The fibroblastic

NIH-3T3 cell line was grown in DMEM and 10% donor calf serum. Tissue adipocytes were isolated from inguinal fat pads of 1-month-old lean male Zucker rats by collagenase digestion [29].

Northern-blot analysis

RNA was extracted from cultured cells as described by Cathala et al. [30]. Total RNA (10 μ g) was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde and transferred on to nylon membranes (Nylon-plus; Schleicher and Schuell, Ecqueville, France). Methylene Blue staining of blots was carried out to ensure the similarity in RNA loading. Hybridization to probes labelled by random priming (Stratagene) was carried out as described by Church and Gilbert [31]. Final washing was performed in $0.2 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS for 15 min at 60 °C.

The linearized pBluescript-Id3 full-length cDNA and an Id2 fragment cloned in a pGEMT-easy vector (Promega) were used as probes [22]. The ADD1 probe was an *EcoRI* fragment excised from the pSVSPORT1-ADD1 expression vector [16]. pFAS-7 has been described previously [32].

Transcription/translation *in vitro* and gel mobility-shift assay

Plasmid DNAs were linearized with the appropriate restriction enzyme and 1 μ g was used as template for transcription/translation *in vitro* using the TNT[®]-coupled reticulocyte lysate system (Promega) under conditions suggested by the manufacturer. In order to estimate transcription/translation efficiency from the different plasmid templates, L-[³⁵S]methionine was included in separate reactions and translated proteins were analysed by SDS/PAGE. The DNA probe used in the gel mobility-shift experiments was a double-strand oligonucleotide derived from wild-type sequence found between nucleotides –94 and –53 relative to the transcription start site in the FAS promoter: 5'-GCCTGGGCGGCGCAGCCAAGCTGTCAGC-CCATGTGCGTGGC-3'. Only the upper strand is shown, and the E-box sequence is underlined. Oligonucleotide was end-labelled using [γ -³²P]ATP and T4 polynucleotide kinase, gel purified, and 50–100 fmol (typically 30 000 c.p.m.) were used as a probe in 20- μ l binding reactions containing 20 mM Tris/HCl, pH 7.5, 5% glycerol, 0.02% Nonidet P-40, 100 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂ and 1 μ g of poly(dI-dC). Reticulocyte lysates of the *in vitro*-translated proteins were mixed and incubated in the presence of the radiolabelled oligonucleotide for 20 min at 37 °C. Samples were loaded on to a non-denaturing 5% polyacrylamide gel. Gels were dried and autoradiographed.

Immunoprecipitation

In vitro-translated [³⁵S]-labelled ADD1/SREBP-1c (5 μ l) and *in vitro*-translated unlabelled Id2 or Id3 (7.5 μ l) obtained from the TNT system (see above) were mixed and incubated at 37 °C for 20 min. The volumes were then made up to 150 μ l with buffer A containing 10 mM Tris/HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.25% Nonidet P-40 and 1% aprotinin, and 1 μ g of an anti-Id2 or anti-Id3 antibody (Santa Cruz). To assess the specificity of the co-immunoprecipitation assay, the same extracts were also incubated with an irrelevant anti-*egr1* rabbit polyclonal antibody (Santa Cruz). As negative controls, anti-Id2 or anti-Id3 antibodies were also added to *in vitro*-translated [³⁵S]-labelled ADD1/SREBP-1c alone or to [³⁵S]-labelled transcription/translation lysate derived from the empty expression plasmid alone. Samples were rocked for 3 h at 4 °C, followed by the addition of a 20- μ l volume of Protein A-agarose beads (Santa Cruz), and the mixtures were incubated for an additional 1-h

period at 4 °C. Beads were pelleted, washed four times in buffer A, heated at 95 °C for 5 min and mixed with an SDS-gel sample buffer consisting of 125 mM Tris/HCl, pH 6.8, 50 % glycerol, 2 % SDS, 5 % β -mercaptoethanol and 0.01 % Bromophenol Blue. Samples were loaded on to a 12 % polyacrylamide gel and subjected to SDS/PAGE. The gel was fixed in 10 % acetic acid/25 % isopropanol, soaked in Amplifier (Amersham), dried and autoradiographed.

Transient cell transfection and CAT assays

For transfection experiments involving tissue adipocytes, cells were washed three times, resuspended in DMEM and 200 μ l of cell suspension (0.5×10^6 cells per assay) were added into a 0.4-cm-gap electroporation cuvette (Eurogentec, Seraing, Belgium) containing 20 μ l of plasmid DNAs including FAS-CAT, RSV- β -gal and the HLH-expressing vectors. To circumvent a non-specific effect of plasmid DNA on transfection efficiency, appropriate amounts of empty pcDNA3 plasmid were added to the indicated expression vectors to obtain an equal total DNA content in all electroporation assays. Electroporation was performed at a voltage of 200 V and a capacitance of 960 μ F using a gene-pulser apparatus (Bio-Rad) [26]. Cells were then transferred into 2-ml Eppendorf tubes containing 1.5 ml of DMEM supplemented with 1 % fetal calf serum. After a 48-h incubation period, adipocytes were washed in PBS, harvested and lysed by three rounds of freezing and thawing in 250 mM Tris/HCl (pH 8.0)/5 mM dithiothreitol. After centrifugation at 12000 g for 15 min at 4 °C, aliquots of the cell lysate (supernatant) were used to determine CAT and β -galactosidase activities as described previously [26]. Another aliquot was withdrawn to test protein content by the method of Lowry et al. [33]. CAT-specific activities were expressed in terms of nmol of chloramphenicol transformed in its butyrylated products/h per mg of cell-lysate protein. Normalized activities are given as the ratio of CAT/ β -gal (as absorbance at 574 nm) to correct for minor variations in transfection efficiencies. Each experiment was repeated at least three times with two independent plasmid preparations. Results are presented as means $\pm$ S.E.M. The level of significance between groups was assessed using either paired or unpaired Student's t tests.

3T3-L1 preadipocytes and adipocytes or NIH-3T3 cells were harvested after trypsinization and resuspended in DMEM. Cell suspension [200 μ l; $(1.5\text{--}2.5) \times 10^6$ cells per assay] was electroporated under conditions similar to those described for isolated rat adipocytes. Cells were seeded in three independent 60-mm-diameter dishes in DMEM (containing 25 mM D-glucose) supplemented with 10 % fetal calf serum and were maintained in a humidified atmosphere of air/CO₂ (9:1, v/v) at 37 °C. After a 48-h culture, cells were washed in PBS, and cell lysates were prepared and processed as described above for CAT activity.

RESULTS

Levels of Id2, Id3, ADD1/SREBP-1c and FAS mRNA in isolated rat adipocytes and in 3T3-L1 cells: modulation during early adipocyte differentiation and by cAMP signalling pathway

We first established the expression profiles of Id2, Id3, ADD1 and FAS mRNAs during the early steps of adipose differentiation of 3T3-L1 cells. As illustrated in Figure 1, a high expression of Id2 and Id3 was observed at cell confluence, whereas ADD1/SREBP-1c and FAS mRNAs were emerging in differentiating cells. In fact, there was a significant overlap in Id and ADD1/SREBP-1c mRNA expression from confluence up to day 2. Importantly, we observed a 2-day lag period between the

Figure 1 Pattern of Id2, Id3, ADD1/SREBP-1c and FAS mRNA expression during early differentiation of 3T3-L1 preadipocytes

At the indicated times relative to confluence (day 0), total RNA was extracted, electrophoresed (10 μ g per lane), blotted on to nylon membranes, and hybridized to the indicated radiolabelled probes (see the Materials and methods section for details). Methylene Blue staining of ribosomal RNA was performed to assess the equivalence in the amounts of loaded RNA. Sizes of the transcripts are indicated in the right margin.

Figure 2 cAMP-induced up-regulation of Id mRNAs in mature 3T3-L1 adipocytes and in isolated rat adipocytes

3T3-L1 8-day post-confluent adipocytes were cultured in the presence of 10 % fetal calf serum and 170 nM insulin. Then, 24 h after the last medium change, cells were exposed or not (control, C) to 10 μ M (–)-isoproterenol (Iso)/100 μ M methyl-isobutylxanthine (MIX) for 1 h. After collagenase digestion, isolated rat adipocytes were maintained for 24 h in the culture medium described above, then treated or not with 10 μ M forskolin (FSK) for 1 h. RNA was then extracted and tested by Northern-blot analysis as described in Figure 1.

Figure 3 Effect of Id2 and Id3 on ADD1/SREBP-1c (A) or USF (B) binding to the FAS promoter

In vitro-translated lysates programmed for synthesis of ADD1 (A) or USF (B) were incubated with a [³²P]-labelled FAS oligonucleotide with or without prior incubation with increasing inputs (in μ l) of Id2-, Id3- or dominant negative ADD1 (ADD1-DN)-programmed lysates (see the Materials and methods section). The amounts of lysate were adjusted according to estimated relative efficiencies of ADD1 and Id plasmids in the transcription/translation system. Unprogrammed lysate (control) was used as a negative control and generated non-specific binding shown by an asterisk. ADD1-DN [16] served as a positive control for suppression of ADD1 binding. Arrows indicate the positions of ADD1 (A) and USF (B) binding complexes.

emergence of ADD1/SREBP-1c (day 1) and that of FAS mRNA (day 3). During this delay, expression of Id2 and Id3 was still observed, suggesting that the expression of FAS, a well-characterized ADD1/SREBP-1c target gene, could be postponed as long as Id proteins are present at significant levels.

Since Id gene expression culminated before cell commitment and dramatically dropped in differentiating cells, we tested whether Id2 and Id3 mRNA levels could be re-induced in mature adipocytes. Thus we evaluated the modulation of Id2 and Id3 gene expression in response to cAMP rising agents. Figure 2 shows that in day-8 post-confluent 3T3-L1 adipocytes, a treatment for 1 h by the combination of (–)-isoproterenol and methyl-isobutylxanthine strongly induced both Id2 and Id3 mRNA levels. During this short interval, the levels of ADD1/SREBP-1c and FAS transcripts remained constant. Likewise, a similar pattern of *Id* gene response was observed in isolated rat adipocytes exposed for 1 h to forskolin. Our results are in agreement with a previous report of a cAMP-induced Id3 gene expression in fibroblasts [9].

Thus the above data show that Ids and ADD1/SREBP-1c can co-exist in the same cell in several physiological situations involving both differentiating preadipocytes and mature adipocytes. On the basis of such results, we investigated further

the putative interaction between these two subclasses of HLH factors.

Id2 and Id3 inhibit binding of ADD1/SREBP-1c to the FAS promoter

As a first step, we examined the ability of Id2 and Id3 to bind ADD1/SREBP-1c and consequently to affect the DNA binding of ADD1/SREBP-1c. All these factors were generated by transcription and translation *in vitro* and used in gel-shift experiments (see the Materials and methods section). As shown in Figure 3(A), Id2 or Id3, as well as unprogrammed lysates, generated a dose-dependent non-specific binding to the probe (lanes 1, 2 and 14). Otherwise, wild-type ADD1 strongly bound to the FAS promoter fragment (arrow, lanes 3, 6 and 9). The previously described dominant negative form of ADD1 (ADD1-DN) [18], which produces a dimer unable to bind DNA, was used as a positive control to abolish the binding of ADD1 (lanes 4 and 5). Overall, *in vitro*-translated Id2 and Id3 prevented ADD1 binding to the E-box oligonucleotide (lanes 7, 8, 10 and 11) as ADD1-DN. Moreover, this effect of Id2 or Id3 occurred in a dose-dependent manner and was quite specific, since an unprogrammed lysate did not affect ADD1 binding (lanes 12 and 13).

Figure 4 Co-immunoprecipitation of *in vitro*-synthesized Id and ADD1/SREBP-1c proteins

In vitro-translated unlabelled or radiolabelled Id2, Id3 or ADD1/SREBP-1c was immunoprecipitated by anti-Id2 or anti-Id3 antibodies, or by an irrelevant anti-*erg1* antibody (I.A.), and analysed by SDS/PAGE (see the Materials and methods for details). The gel was fixed, soaked in fluorographic enhancing solution, dried and exposed to Kodak X-ray film for 5 days. Arrows on the right indicate the positions of the ADD1/SREBP-1c proteins. The positions of the molecular-mass markers (in kDa) are shown on the left. Lane 1, pattern of [³⁵S]-labelled ADD1/SREBP-1c-translated products; lanes 2 and 3, absence of cross-reactivity of [³⁵S]ADD1/SREBP-1c proteins with anti-Id2 and anti-Id3 antibodies; lanes 4 and 5, specific immunoprecipitation of [³⁵S]ADD1/SREBP-1c from the ADD1/SREBP-1c–Id2 complex by an anti-Id2 antibody; lanes 6 and 7, specific immunoprecipitation of [³⁵S]ADD1/SREBP-1c from the ADD1/SREBP-1c–Id3 complex by an anti-Id3 antibody.

The specificity of the interaction described here between ADD1/SREBP-1c and Id proteins was further established by examining the effects of Id2 and Id3 on the binding of USF, another bHLH-LZ factor, also known to *trans*-activate the FAS promoter through the binding to the same DNA-binding site [20,34]. Rabbit reticulocyte lysates were programmed with an USF vector and similar gel-shift experiments were performed.

Results in Figure 3(B) show that USF binding to the FAS probe was not affected by ADD1-DN (lanes 2–4). This agrees with data from Wang and Sul [20,34] showing that ADD1/SREBP-1c and USF are not able to dimerize. In addition, no inhibition of USF binding to the probe was observed with Id2 (lanes 9 and 10), whereas a slight decrease was noted with Id3 (lanes 6 and 7). The absence of effect of Id2 on the formation of USF–DNA complexes has to be related with previous studies that demonstrate no interaction between USF and Id2–glutathione S-transferase fusion proteins [11]. Taken together, these results suggest that ADD1/SREBP-1c is a privileged partner for Ids.

Direct interaction of ADD1/SREBP-1c and Id proteins

To establish the interaction between Ids and ADD1/SREBP-1c in the absence of DNA, a co-immunoprecipitation approach was also used. ADD1/SREBP-1c labelled with L-[³⁵S]methionine and unlabelled Id2 or Id3 proteins were generated separately by transcription/translation *in vitro*. Radiolabelled ADD1/SREBP-1c was then incubated with Id2 or Id3, and immunoprecipitated in the presence of commercially available anti-Id2 or anti-Id3 antibodies. The products were analysed on a polyacrylamide gel (Figure 4). Preliminary data indicated that *in vitro*-translated [³⁵S]-labelled Id2 and Id3 were recognized specifically by the anti-Id2 and anti-Id3 antibodies, respectively, but not by an irrelevant antibody. Moreover, these antibodies did not cross-react with any labelled protein in the unprogrammed lysate (results not shown). Transcription/translation of the pSVSPORT1-ADD1 plasmid generated one major and two minor polypeptide species with apparent molecular masses ranging from 35 to 45 kDa (lane 1). These multiple translation products are likely to be the result of alternative stop codons contained in pSVSPORT1 and are not produced when the empty vector is used (results not shown). No labelled product could be recovered by anti-Id2 and anti-Id3 antibodies from ADD1-programmed lysate (lanes 2 and 3). Importantly, the preincubation of the Id3 lysate in the presence of [³⁵S]-labelled ADD1/SREBP-1c allowed the precipitation of ADD1 translation products (lane 7), whereas an irrelevant antibody was inefficient (lane 6). Likewise, addition of an anti-Id2 antibody to a mixture containing both [³⁵S]-labelled ADD1/SREBP-1c and non-radioactive Id2 immunoprecipitated ADD1 proteins, but to a lesser extent (lane 5), whereas an irrelevant

Figure 5 Inhibition of FAS-CAT activity in isolated rat adipocytes overexpressing Id2 (A) or Id3 (B) proteins

Isolated rat adipocytes (0.5×10^6) were co-transfected with 20 μg of the FAS-CAT reporter gene, 5 μg of RSV-β-gal and the indicated amounts of Id-expressing and empty vectors up to 30 μg. To correct DNA size charge effects, all electroporation assays contain the same amounts of plasmid DNA. Normalized CAT/β-galactosidase activities are expressed as the percentage of CAT activity measured in control cells co-transfected with the reporter gene and the empty expression plasmids (see the Materials and methods section for details). Values represent means ± S.E.M. of at least three independent transfection experiments using distinct plasmid preparations. * $P < 0.05$; ** $P < 0.001$, Id-transfected versus control cells.

Figure 6 Inhibition of FAS-CAT activity in 3T3-L1 adipocytes overexpressing Id2 (A) or Id3 (B) proteins

3T3-L1 day-8 post-confluent adipocytes ($1.5\text{--}2.5 \times 10^6$) were transfected with a procedure identical to that described in Figure 5. Results are expressed as the percentage of the normalized CAT activity measured in control cells and represent the means $\pm$ S.E.M. of at least three separate experiments. * $P < 0.01$; ** $P < 0.001$, Id-transfected versus control adipocytes.

Figure 7 Co-transfection of an Id3 antisense plasmid further increases ADD1-stimulated FAS-promoter activity in 3T3-L1 preadipocytes

At confluence (day 0), 3T3-L1 preadipocytes were harvested and 2×10^6 cells were co-transfected by electroporation with 10 µg of the FAS-promoter reporter gene, 5 µg of RSV-β-gal and either 30 ng of the ADD1 expression vector or 100 ng of the Id3 antisense (AS) vector, or a combination of both. Empty expression plasmids were added to ensure equal amounts of DNA in all electroporation assays. A FAS-CAT plasmid mutated in the E-box was used as a negative control to assess the specificity of ADD1-induced transactivation. CAT activities were measured as mentioned in Figure 5. Results are expressed as the percentage of control preadipocytes and represent the means $\pm$ S.E.M. of four independent experiments. * $P < 0.001$, ADD1- or ADD1- and Id3 antisense-transfected cells versus control cells; § $P < 0.05$, ADD1- and Id3 antisense-transfected cells versus cells transfected with ADD1 alone.

antibody did not (lane 4). Thus these experiments demonstrate that Id2 and ADD1/SREBP-1c or Id3 and ADD1/SREBP-1c can interact physically with each other to form a heterodimer *in vitro* in the absence of DNA. Since it has been shown that other factors of the bHLH-LZ family do not interact with Id2 [11], ADD1/SREBP-1c appears to behave as an unusual member of this subclass.

Id2 and Id3 inhibit *trans*-activation of the FAS promoter in isolated rat adipocytes and in cultured mouse 3T3-L1 adipocytes

On the basis of interactions *in vitro* between Ids and ADD1/SREBP-1c demonstrated above, we first tested whether overexpression of Ids in adipocytes could affect the FAS gene activity, the expression of which is ADD1-dependent [16,18,35]. Id2 or Id3 expression vectors were thus transfected in mature rat

adipocytes, together with a CAT reporter gene whose expression was driven by an FAS promoter containing the transcriptionally active ADD1/SREBP-1c-binding site. To circumvent non-specific effects of increasing amounts of plasmid DNA on reporter gene expression, empty DNA vector was added to equalize DNA content in all electroporation assays. In addition, CAT activities were normalized for minor variations in transfection efficiency using an RSV-β-gal co-transfected plasmid as an internal control. Results in Figure 5 show that overexpression of Id2 or Id3 in rat adipocytes was able to significantly repress FAS-promoter-driven CAT activity in a dose-dependent manner.

Then, co-transfection experiments were conducted in the 3T3-L1 cell line, taking advantage of the fact that various equilibria between Id and ADD1/SREBP-1c proteins were achieved in cells harbouring different phenotypes. In 3T3-L1 adipocytes (8-day post-confluence), as described above in mature rat

Figure 8 Inhibition of ADD1-stimulated *FAS* promoter activity by Id2 and Id3 expression vectors in NIH-3T3 fibroblasts

Quiescent NIH-3T3 fibroblasts were transfected as described above with 10 μ g of the *FAS* promoter reporter gene, 5 μ g of RSV- β -gal and ADD1 (30 ng) and/or Id (20 μ g) expression vectors, as indicated at the bottom of the Figure. Empty expression plasmids were added to obtain identical amounts of DNA in transfection assays. Results are expressed as the percentage of control NIH-3T3 cells and represent the means $\pm$ S.E.M. of three independent experiments. * P < 0.05, versus cells transfected with empty vectors; § P < 0.05, versus cells transfected with ADD1 alone.

adipocytes, increasing amounts of Id2 and Id3 vectors led to reduced *FAS*-promoter-driven CAT activity compared with that measured in cells transfected with an empty vector (Figure 6). The ability of Id2 and Id3 to repress the *FAS*-promoter activity appeared stronger in 3T3-L1 than in isolated rat adipocytes. This might reflect either a higher transfection efficiency in 3T3-L1 adipocytes, or a difference in Id2 and Id3 endogenous levels between the two cellular systems. Together, these results demonstrate for the first time that, in adipocytes, Id2 and Id3 proteins are able to repress *FAS* gene transcription.

In another set of experiments conducted in preadipocytes harbouring high levels of Ids relative to those of ADD1/SREBP-1c, we tested the ability of Id to modulate ADD1-dependent transcription of the *FAS* gene. An Id3 antisense expression plasmid was thus co-transfected with an ADD1/SREBP-1c expression vector in 3T3-L1 preadipocytes (day 0 relative to confluence). An empty expression vector was used as a control for specificity; thus the observed effects resulted from cloning the insert in the antisense orientation. As expected, ADD1/SREBP-1c was able to stimulate *FAS*-CAT activity (Figure 7). This effect was mediated by the target sequence for ADD1/SREBP-1c on the *FAS* promoter, since the mutation within this site abolished the *FAS*-promoter response to ADD1/SREBP-1c. Overall, *FAS*-promoter-driven CAT activity was higher in cells transfected with both ADD1/SREBP-1c and Id3 antisense vectors than in cells transfected with ADD1/SREBP-1c vector alone.

Functional antagonism between Ids and ADD1/SREBP-1c on the *FAS* promoter in NIH-3T3 fibroblasts

To definitely establish the functional antagonism between Ids and ADD1/SREBP-1c, we used NIH-3T3 cells, a fibroblastic cell line non-permissive for adipose conversion. In these cells, ADD1/SREBP-1c and *FAS* gene products were virtually absent [16]. Moreover, since these experiments were performed in post-confluent cells, endogenous Id levels were strongly down-regulated. Under these conditions, the *FAS*-CAT reporter gene

was co-transfected together with Id and ADD1 expression vectors. ADD1/SREBP-1c alone strongly stimulated *FAS*-promoter activity (Figure 8), in agreement with many studies [18,35–37]. Importantly, we found that co-transfection of Id2 or Id3 was able to repress ADD1/SREBP-1c-stimulated *FAS*-promoter activity by 82 and 46 %, respectively. These results demonstrate that when expressed ectopically, Ids and ADD1 can functionally interact *in vivo* to modulate *FAS* promoter activity.

DISCUSSION

Id proteins can be viewed as positive regulators of proliferation and negative regulators of differentiation even if some aspects of their expression may vary depending on the cell type (for a review see [1]). They abrogate the function of bHLH proteins through the formation of inactive heterodimers. The precise physiological role of different Id proteins still remains to be clarified in the developmental process of adipogenesis [22,38]. A previous study from our laboratory has demonstrated that Id3 inhibits the adipose conversion of 3T3 cells [22]. However, putative partners of the bHLH or of the related bHLH-LZ have not yet been identified in the adipocyte. The present study presents evidence for the functional interaction of Id2 and Id3 with ADD1/SREBP-1c, a transcriptional factor belonging to the bHLH-LZ family that activates several major genes of adipocyte metabolism and development [12,15,16,18]. In the adipose cell, the modulation of Id levels according to developmental or environmental conditions could in turn regulate ADD1/SREBP-1c activity, and thus the adipocyte lipogenic pathway.

So far, bHLH-LZ proteins have been shown to be unaffected by the Id1 and Id2 proteins [11]. Thus the ubiquitous c-Myc involved in adipose precursor proliferation and differentiation [39], USF protein, a cellular factor involved in transcription of the *FAS* gene [34], AP4 protein, the *trans*-activator binding to the AP4 site of SV40 enhancer [40], and TFE3 protein, a transcription activator that binds to the enhancer of immunoglobulin heavy chain gene [41], do not dimerize with Id1 and Id2 proteins [11]. The reason for the inability of Id1 and Id2 proteins to inhibit this set of bHLH-LZ proteins could arise from the incompatibility of the HLH motifs in the Id and the bHLH-LZ proteins. Structural domains of Id proteins may confer various ways for interaction with other bHLH factors. The HLH domain of the Id proteins is highly conserved [42,43], but there are however several amino acid substitutions in the loop and second helical regions of Id3 that differ from other Ids. This could be at the basis of the slight inhibitory effect of Id3 observed on the formation of USF-DNA complexes, whereas Id2 has no effect. Thus ADD1/SREBP-1c is unique in its ability to interact with Id proteins. Further studies out of the scope of the present work are needed to address the structural basis of the interaction of Id2 and Id3 with ADD1/SREBP-1c. Whatever the exact regions involved in the dimerization between Id and ADD1/SREBP-1c proteins, the present study describes an original and functional interaction between a bHLH-LZ and a dnHLH protein.

It is now well established that the E-box sequence around nucleotide –59 exerts a tight control on *FAS* gene transcription [19,25]. Whereas ADD1/SREBP-1c efficiently up-regulates *FAS* gene expression through this site, another factor, the ubiquitously expressed USF, also exhibits potent *trans*-activating properties. USF, like ADD1/SREBP-1c, has been involved in the stimulation of the *FAS* gene by insulin [18,20]. Our data indicate that Id3 interacts poorly with USF *in vitro*, whereas Id2 does not. Since USF and ADD1/SREBP-1c bind to the *FAS* promoter in a mutually exclusive manner [20], the differential interaction of Id2/Id3 with ADD1/SREBP-1c or USF could alter their re-

spective recruitment to the FAS promoter. This hypothesis is quite consistent with our experimental data that indicate a residual functional activity of the *FAS* gene in adipocytes overexpressing Id2 or Id3. This residual activity might reflect the involvement of the endogenous USF and/or other unknown auxiliary *trans*-activators. In this regard, it is noteworthy that targeted disruption of the SREBP-1 gene generates knock-out mice with a normal development of adipose tissue and with normal amounts of several adipocyte-specific mRNA, including FAS [17]. Whereas other *trans*-factors may also contribute to *FAS* gene expression [44,45], the functional studies described here suggest a privileged dimerization of Id2 and Id3 with ADD1/SREBP-1c *in vivo*.

This study underlines the important and redundant function of Id proteins in regulating the expression of the *FAS* gene in adipocytes, through their interaction with ADD1/SREBP-1c. It is reasonable to think that the expression of the constantly expanding family of ADD1/SREBP-1c target genes such as those encoding for glycerol-3-phosphate acyltransferase [46], S14 [47], stearoyl CoA desaturase [48] or leptin [18], could also be modulated by the levels of Id proteins. During the course of adipose differentiation, it is likely that the equilibrium between Id and ADD1 protein levels plays a key role in the expression of genes activated by ADD1/SREBP-1c. In the preadipocyte, the high content of Id2 and Id3, together with the low levels in ADD1/SREBP-1c, prevent the cell from expressing ADD1/SREBP-1c-activated genes. Conversely, during adipose conversion, both the rapid and sharp decrease in Id2 and Id3 abundance and the marked increase in ADD1/SREBP-1c levels potentiate the expression of the target genes. Moreover, the present work shows that, in mature adipocytes, Id levels can be strongly and rapidly modulated by environmental conditions. In this regard, activation of the β -adrenergic-receptor signalling pathway results in an immediate and strong up-regulation of *Id2* and *Id3* gene expression. The levels of intracellular cAMP could thus represent a basic mechanism to control the equilibrium between Ids, ADD1/SREBP-1c and Ids-ADD1/SREBP-1c heterodimers, and consequently to modulate the expression of genes *trans*-activated by ADD1/SREBP-1c. Besides Id abundance, intrinsic activity of dominant negative factors might also be modulated at a post-transcriptional level. In this regard, phosphorylation alters the ability of Id proteins to heterodimerize and to sequester other HLH factors [49,50]. Thus, it will also remain to investigate whether Id phosphorylation could control the interaction with ADD1/SREBP-1c. In a more general context, one can consider that variations *in vivo* in Id levels and/or activity may be an additional mechanism for control of ADD1 activity operating in concert with gene transcription and proteolytic processing.

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