Liver-enriched Inhibitory Protein (LIP) Actively Inhibits Preadipocyte Differentiation through Histone Deacetylase 1 (HDAC1)*

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Houssain-Salem Abdou†, Ella Atlas§, and Robert J. G. Hache¶

From the †Graduate Program in Biochemistry, University of Ottawa, Ottawa, Ontario K1N 6N5, the ‡Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario KIA OK9, and the †Department of Anatomy and Cell Biology, Southern Alberta Cancer Research Institute, University of Calgary, Calgary, Alberta T2N 1N4, Canada

The CCAAT/enhancer-binding protein β (C/EBPβ) is expressed as three isoforms (LAP*, liver-enriched activating protein (LAP), and liver-enriched inhibitory protein (LIP)) that differentially regulate gene expression. The interplay between LAP*, LAP, and LIP in regulating cellular processes is largely unknown, and LIP has been largely regarded to repress transcription through a passive heterodimerization-dependent mechanism. Recently, we have shown that p300/GCN5 and mSin3A/HDAC1 differentially regulate the ability of C/EBPβ to stimulate preadipocyte differentiation through activation of C/ebpα transcription. Here, we have mapped requirements for binding of mSin3A/HDAC1 to LAP/LAP* and LIP to a 4-amino acid motif in the central region of LAP/LAP* (residues 153–156) and the N terminus of LIP. Reducing mSin3A/HDAC1 binding to LAP/LAP* and LIP through deletion of this motif reduced the recruitment of HDAC1 to the C/ebpα promoter and increased preadipocyte differentiation stimulated by insulin and 1-methyl-3-isobutylxanthine. Additional studies showed that the interaction of HDAC1 with LIP provides for active repression of C/ebpα transcription and is largely responsible for the ability of LIP and HDAC1 to repress preadipocyte differentiation. Thus, although mSin3A/HDAC1 interacted readily with LAP/LAP* in addition to LIP and that expression of LAP/LAP* was sufficient to recruit HDAC1 to the C/ebpα promoter, mutations in C/ebpβ that abrogated HDAC1 association to LAP/LAP* in the absence of LIP provided no additional stimulation of differentiation or transcription beyond the deletion of LIP alone. The implication of these results for the interaction between p300/GCN5 and mSin3A/HDAC1 in regulating C/EBPα transcription and preadipocyte differentiation are discussed.

The CCAAT/enhancer-binding protein β (C/EBPβ) is a basic leucine zipper (bZip) transcription factor that plays key roles in cellular differentiation, proliferation, stress responses, and inflammation. The C/ebpβ gene encodes three isoforms, LAP* (liver-enriched activating protein*), LAP, and LIP (liver-enriched inhibitory protein) translated from a single mRNA from three consecutive in-frame methionines (Fig. 1A) (1, 2). Relative expression of LAP*/LAP/LIP varies according to cell type, and the three isoforms dimerize interchangeably and heterodimerize broadly with other bZip factors. The shorter LIP isoform is distinguished by the absence of the transcriptional activation functions that occur in the N terminus of LAP*/LAP. Thus, LIP has been primarily hypothesized to be a passive repressor of transcription that reduces transcriptional activation through competition for DNA-binding sites and the formation of heterodimers in which only one partner contributes to activation (1). Although the relative level of LAP/LAP* in a cell is regulatable, generally the level of LIP expression is approximately equivalent to the level of LAP, with LAP* levels usually being significantly lower (2).

C/EBPβ has been shown to play an important role at the onset of the differentiation of preadipocytes to white adipose tissue. In culture, preadipocytes respond to adipogenic stimulus by rapidly inducing C/EBPβ and C/EBPδ, whereas in vivo deletion of C/ebpβ together with the C/ebpδ gene compromises adipose tissue development (3). C/EBPβ acquires transcription competence 16–24 h following exposure of preadipocytes to adipogenic stimulus and directly induces the commitment factors C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) that drive differentiation to completion (4–6). Glucocorticoid hormones, key metabolic regulators, enhance adipogenesis by increasing the percentage of cells that commit to differentiation (7). This stimulatory effect of glucocorticoids is mediated, at least in part, by increasing the transcriptional effectiveness of C/EBPβ (5, 6).

A number of factors regulate the transcriptional activation potential of C/EBPβ in stimulated preadipocytes. C/EBPζ (or CHOP10), a C/EBP family member compromised for DNA binding, initially heterodimerizes with C/EBPβ to keep it from binding to transcriptional response elements (8, 9). C/EBPζ expression is down-regulated in 14–18 h following adipogenic stimulation, freeing C/EBPβ to bind DNA (9). Concurrently, DNA binding of C/EBPβ is facilitated by MAPK phosphorylation beginning at 4 h post-stimulation and GSK3β phosphorylation ~14 h into differentiation (4).

Moreover, we have shown that the ability of C/EBPβ to activate C/ebpα expression in preadipocytes stimulated to differentiate is initially reduced through the interaction of C/EBPβ with an mSin3A/histone deacetylase 1 (HDAC1) complex that...
dampens C/EBPβ acetylation within a cluster of three lysines between amino acids 98 and 102 of the LAP/LAP* isoforms and acetylation of histones at the C/ebpα promoter (5, 6). The effect of glucocorticoids in stimulating preadipocyte differentiation is accomplished, at least in part, through titration of mSin3A/HDAC1 from C/EBPβ during the first 24 h of differentiation. Displacement of mSin3A/HDAC1 correlates with p300/C/EBPβ acetylation of histone H4 at the C/ebpα promoter, and enhanced recruitment of RNA polymerase II (5, 6, 10).

A second member of the C/EBP family of transcription factors, C/EBPδ, has also been shown to be regulated by an mSin3A/HDAC1 corepressor complex that is sensitive to steroid (11). Furthermore, titration of corepressor complexes from transcriptional activators at the onset of differentiation is known to occur more generally. To cite just one example, the basic helix loop helix transcription factor MyoD is similarly activated at the onset of skeletal myogenesis through a differentiation medium-dependent displacement of HDAC1 followed by increased histone acetylation (12, 13).

In this study, we sought to increase definition of the mechanism through which mSin3A/HDAC1 acts to repress C/EBPβ-mediated transcription in preadipocytes. Our results identified amino acids 153–156 of C/EBPβ as being required for association of LAPI/LAP/LIP with mSin3A/HDAC1. Deletion of amino acids 153–156 from the three C/EBPβ isoforms enhanced C/EBPα expression and the differentiation of NIH 3T3 fibroblasts and 3T3 L1 preadipocytes into mature adipocytes. Overexpression of LIP reduced preadipocyte differentiation and the repression of C/ebpα expression in a manner that was compromised by disruption of its association with HDAC1. Remarkably, compromising HDAC1 association with LAP/LAP* had little further effect on their transcriptional activation potential or ability to stimulate preadipocyte differentiation beyond what was obtained by deletion of LIP. These results provide the first evidence that transcriptional repression through LIP occurs through an active HDAC1-dependent mechanism and indicate that the effects of mSin3A/HDAC1 on C/EBPβ and preadipocyte differentiation are primarily dependent on LIP. They also elaborate a mechanism of transcriptional control of C/EBPβ that is likely to be relevant to its actions as a key factor in the differentiation of a number of tissues.

**EXPERIMENTAL PROCEDURES**

**Constructs**—Murine C/EBPβ cDNA and the external deletion constructs (C/EBPβ153–156, N107, N140, N168, N184, N217) were amplified by PCR using the Vent DNA polymerase (New England Biolabs) and subcloned into the pGEX2T plasmid (GE Healthcare) for bacterial GST fusion protein expression. pGEX2T-C/EBPβ151–156 and pGEX2T-C/EBPβ141–166 were produced by a reverse PCR using PFU turbo DNA polymerase (Stratagene) and pGEX2T-C/EBPβ as a template. Moreover, the C/EBPβ cDNA was subcloned into the pcDNA3.1(−) (Invitrogen) and pLXSN plasmids (Clontech) for the transient transfection and the viral infection experiments, respectively. pcDNA3.1(−)/C/EBPβ153–156 and pLXSN-C/EBPβ153–156 were constructed using PFU turbo DNA polymerase (Stratagene) using pcDNA3.1(−)/C/EBPβ and pLXSN-C/EBPβ as template, respectively. The pcDNA3.1(−)/LIP (WT or 6C) and pLXSN-LIP (WT or 6C) constructs were generated by amplifying the LIP cDNA from pcDNA3.1(−)/C/EBPβ or pcDNA3.1(−)/C/EBPβ153–156, respectively, and by subcloning them into either pcDNA3.1(−) or pLXSN vectors. The HDAC1, GR, Renilla luciferase, and C/EBPs luciferase reporter (−350/−7) constructs have been described previously (5, 6).

**Cell Culture, Retroviral Infection, and Cellular Differentiation**—COS7 cells (American Type Culture Collection (ATCC)) were maintained in DMEM containing 4.5 g/liter glucose and 10% fetal bovine serum. NH 3T3 and 3T3 L1 cells (both from ATCC) were maintained in DMEM containing 4.5 and 1.5 g/liter glucose, respectively, supplemented with 10% calf serum. Replication-incompetent pLXSN-based retroviruses were generated in Phoenix Amphi packaging cells (from G. Noland, ATCC) and used to infect 50–70% confluent 3T3 L1 and NIH 3T3, as described previously (5, 6). Cells were selected with the geneticin antibiotic (0.4 mg/ml) (Invitrogen) for 10 days.

For the differentiation assay, 2 days of postconfluent NIH 3T3 or 3T3 L1 cells were treated with either MIX (500 μM) and insulin (100 nM) (MI), or MIX, insulin, and dex (250 nM) (MID) with their respective media for 48 h. Cells were subsequently incubated in their media supplemented with 100 nM insulin until harvesting as specified in each experiment. Finally, for the differentiation of LIP-expressing 3T3 L1 cells, the same procedure was used but 1 mM MIX and 1 μM dex were used instead to increase the efficiency of differentiation.

**Oil Red O Staining and Western Blots**—Six or 8 days after induction of differentiation of the NIH 3T3 or the 3T3 L1 cells, respectively, cells were fixed with 4% formaldehyde and stained overnight with Oil Red O, as described previously (5, 6). Phase contrast photomicrographs were then taken at the specified magnifications.

For Western analysis, cells were lysed using a standard mammalian whole cell lysis buffer. Western blot analysis was performed using antibodies against C/EBPβ (C19), C/EBPα (14AA), PPARγ (H100), adipin (P16), and actin (H300) (Santa Cruz Biotechnology).

**In Vitro Binding Assay**—In vitro translated (Tnt-coupled reticulocyte lysate system, Promega) and 35S-labeled GCN5 and mSin3A constructs were incubated with 1 μg of GST fusion proteins, expressed in Escherichia coli BL21, for 2 h at 4 °C in 0.6× lysis buffer (25 mM Heps, pH 9.9, 100 mM KCl, 2 mM EDTA, 20% glycerol, 2 mM DTT, and 1 mM PMSF) containing 0.15% Nonidet P-40. After three 1-ml washes using the same binding buffer, proteins were resolved by 10% SDS-PAGE, after which the gels were stained with a Coomassie stain (Sigma) and dried, and binding was visualized by PhosphorImager analysis (GE Healthcare).

**Coimmunoprecipitation Assay**—Eighty percent confluent COS7 cells were transiently transfected with 2 μg of the expression plasmid for FLAG-HDAC1 and 2 μg of the pcDNA3.1(−)/C/EBPβ constructs using the FuGENE HD reagent (Roche Applied Science) for 16 h. The media were changed the next day for an additional 16–24 h. Cells were then lysed using a lysis buffer (150 mM NaCl, 1 mM EDTA, pH 8.0, 50 mM...
Active Repression by LIP Mediated through HDAC1

Tris-HCl, pH 7.4, 1× protease inhibitor mixtures (Sigma) and 0.5% Nonidet P-40 on a nutator for 25 min at 4°C. Whole cell extracts were obtained by spinning the lysed cells at 4°C for 10 min at maximum speed in a bench microcentrifuge. HDAC1 immunoprecipitation was performed in the lysis buffer with only 0.15% Nonidet P-40 for 2 h at 4°C using the M2 FLAG resin (Sigma). Following three 1-ml washes with the same binding buffer, the proteins were resolved in a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with antibodies against C/EBPβ (C19) (Santa Cruz Biotechnology) and M2 FLAG monoclonal (Sigma).

For endogenous HDAC1 immunoprecipitation, the same procedure was applied with the following modifications. 3T3 L1 cells stably expressing the LIP constructs were lysed at confluency and subjected to immunoprecipitation using the HDAC1 (C19) antibody and the Gal4 (DBD) (both from Santa Cruz Biotechnology) as a nonspecific control, followed by a 1-h incubation with protein-G-Sepharose beads (Sigma).

Luciferase Reporter Gene Assay—Eighty percent confluent NIH 3T3 cells were maintained in phenol red-free DMEM (4.5 g/liter glucose) supplemented with 10% charcoal-stripped FBS and were transiently transfected using the FuGENE HD reagent, as per the manufacturer’s instructions. Two hundred nanograms of the C/EBPα-luciferase reporter plasmid, 100 ng of pTL-GR construct (full-length glucocorticoid receptor), 25 ng of the Renilla luciferase reporter plasmid (as an internal control), and either 50 ng of empty pcDNA3.1(−) or with the different C/EBPβ constructs were transfected into the cells. For transcription assays with the LIP isoforms, the same protocol was applied with the addition of 50, 75, and 100 ng of either LIP or LIP6C. Total transfected DNA was brought to 1 μg with the pcDNA3.1(−) plasmid. 16 h after transfection, the media were changed, and the cells were treated with either ethanol (vehicle) or 1 μM dexamethasone. Sixteen hours later, cells were harvested using Passive Lysis Buffer (Promega), and the luciferase activity was measured using a luminometer. Each experiment was performed in duplicate and corrected for transfection efficiency by using the cotransfected Renilla expression plasmid.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assay was performed as described previously (5, 6). Briefly, 2 days post-confluent (day 0) NIH 3T3 cells stably expressing the C/EBPβ constructs were cultured in the presence of MIX and insulin for 24 h. The cells were then incubated with the addition of 50, 75, and 100 ng of either LIP or LIP6C. 16 h after transfection, the media were changed, and the cells were treated with either ethanol (vehicle) or 1 μM dexamethasone. Sixteen hours later, cells were harvested using Passive Lysis Buffer (Promega), and the luciferase activity was measured using a luminometer. Each experiment was performed in duplicate and corrected for transfection efficiency by using the cotransfected Renilla expression plasmid.

Real Time qRT-PCR Analysis—Total RNA was reverse-transcribed according to standard protocol. cDNA was amplified as described previously (5, 6). Regions +835/+1065 of C/EBPβ transcript (forward primer, TGG AGA ACA GGA AGC AG, and reverse primer, CCA TGG CCT TGA CCA AGG AG) and +52/+242 of PPARγ2 transcript (forward primer, GAA ACT CTG GGA GAT TCT CC, and reverse primer, GCT GGA GAA ATC AAC TGT GG) were amplified and normalized to β-actin transcript (forward primer, GAC TTC GAG CAA GAG ATG GC and reverse primer, CCA GAC AGC ACT GTG TTG GC).

RESULTS

Amino Acids 153–156 of C/EBPβ Are Required for mSin3A/HDAC1 Binding—Previously, we have shown that mSin3A directly interacts with C/EBPβ, with HDAC1 association to C/EBPβ mediated through its binding to mSin3A (5). Furthermore, our previous work also implicated the region of C/EBPβ between amino acids 98 and 102 in the regulation of mSin3A as acetylation of lysines within these residues by GCN5 strongly reduced binding (6). Therefore, we initiated our dissection of the interface for mSin3A/HDAC1 binding to C/EBPβ by examining the ability of in vitro translated, 35S-labeled mSin3A to bind to a series of GST–C/EBPβ fusion proteins, with a focus on N-terminal deletions (Fig. 1B).

Somewhat unexpectedly, deletion of the N-terminal 108 residues of C/EBPβ, including the GCN5 acetylation sites, had little if any effect of mSin3A binding. Furthermore, a GST-C/EBPβ construct containing amino acids 1–108 alone (β108C) failed to interact with mSin3A. Thus, it seemed from these results that amino acids 98–102 might not be directly required for mSin3A binding. Rather, the data suggested that acetylation of these residues was more likely to provide for ionic interference with the binding of mSin3A to another site on C/EBPβ. Post-translational modifications within transcription factors influencing interactions distant from the modification site have been described in several instances, including for C/EBPβ (14), and thus our observation, although relatively uncommon, does not establish a precedent. Curiously, initial C-terminal deletions of C/EBPβ did not shed further light on the amino acids that mediated mSin3A binding, as truncation of the bZip domain also disrupted the interaction with mSin3A (Fig. 1B and data not shown).

Although these data suggested that mSin3A association depended on the bZip domain of C/EBPβ, additional experiments with a large internal deletion mutant (Δ121–198) and other constructs indicated that the central region of the protein was also required for binding and suggested that the contribution of the bZip domain to binding was related more to C/EBPβ dimerization than to providing a binding interface (data not shown). These results were consistent with a recent report demonstrating that mSin3A binding to C/EBPβ is dependent on dimerization, but it occurs outside of the bZip domain (11). However, the specific amino acids required for mSin3A binding to C/EBPβ were not determined. Refining our internal truncations of C/EBPβ revealed that deletion of amino acids 141–168 reduced mSin3A binding by ~70%. Furthermore, this reduction in binding was largely maintained by deletion of just the six amino acids between 151 and 156 (Fig. 1C).

As LIP translation is initiated at Met-152 (Fig. 1A), expression in C/EBPβΔ151–156 would not only result in the reduction of mSin3A/HDAC1 binding to C/EBPβ, it would abrogate LIP expression, which occurs at levels approximately equal to the sum of LAP/LAP* expression, both endogenously (Fig. 1D, lane
and from the wild type C/EBP\(_{141-168}\) expression vector we employed in this study (lane 2). This mandated further refinement of the C/EBP\(_{1107-1217}\) truncation prior to testing in vivo. Thus, we prepared C/EBP\(_{1107-1217}\), which retained the LIP initiator methionine. Expression of C/EBP\(_{1107-1217}\) in cells by transient transfection produced the same relative proportions of LAP*, LAP, and LIP as translated from the endogenous C/EBP\(_{1107-1217}\) mRNA, at the same levels as expressed from a WT C/EBP\(_{1107-1217}\) vector (Fig. 1D, lane 3).

In our previous work, we demonstrated that mSin3A interacted with C/EBP\(_{1107-1217}\) with a specific HDAC1-containing complex, and HDAC1 did not interact with C/EBP\(_{1107-1217}\) in the absence of mSin3A (5). Furthermore, given the abundance of HDAC1 and mSin3A in the cell, binding of the mSin3A-HDAC1 complex in vivo to C/EBP\(_{1107-1217}\) was most efficiently revealed in coimmunoprecipitations utilizing FLAG-tagged HDAC1 expressed exogenously at low levels (5). Here, comparing the coimmunoprecipitating C/EBP\(_{1107-1217}\) isoforms LAP*/LAP to FLAG-HDAC1 in

**FIGURE 1.** Amino acids 153–156 and the bZip domain of C/EBP\(_{1107-1217}\) are required for mSin3A binding.

**A,** schematic presentation of the three isoforms of the C/EBP\(_{1107-1217}\). The schema shows the positions of N-terminal activation domains of LAP*/LAP and the bZip domain common to the three isoforms that mediates homo/heterodimerization and DNA binding. GCN5-dependent acetylation of C/EBP\(_{1107-1217}\) has been mapped to a cluster of lysines 98, 101, and 102 that occur in LAP*/LAP (Ac). Additional regulatory domains (RD1 and RD2) proposed by William et al. (30) are also indicated.

**B,** quantification of radioblots of the binding of \(^{35}\)S-labeled in vitro translated mSin3A to a series of GST-C/EBP\(_{1107-1217}\) fusion peptides with N-terminal (108C) or C-terminal (N107 to N217) truncations (n = 3; ± S.D., * = p < 0.05). C, \(^{35}\)S-mSin3A binding to GST-C/EBP\(_{1107-1217}\) with internal deletions of amino acids 151–156 and 141–168 (C/EBP\(_{1107-1217}\) and C/EBP\(_{1107-1217}\)) as in A, with quantification indicated below the radioblot (top) (n = 3; ± S.D., p < 0.01). The lower panel shows a Coomassie stain of SDS-PAGE separation of the input GST fusion peptides. D, COS7 cells were cotransfected with expression plasmids for FLAG-HDAC1 and either C/EBP\(_{1107-1217}\) or C/EBP\(_{1107-1217}\). Whole cell lysates were immunoprecipitated with a FLAG affinity resin, with 10% of input material being shown on the right. Coimmunoprecipitation of LAP*/LAP with FLAG-HDAC1 was quantified as shown (n = 3; ± S.D., p < 0.01). E, Western analysis showing the expression level of exogenous C/EBP\(_{1107-1217}\) constructs expressed by transient transfection in NIH 3T3 cells. IP, immunoprecipitation. F, transient transfection analysis of the transcriptional activation potential of C/EBP\(_{1107-1217}\) and C/EBP\(_{1107-1217}\) at the C/epb promoter (~350–7) measured from a luciferase reporter gene. 100 ng of HDAC1 expression vector was also transfected as indicated. Luciferase activity was corrected for transfection efficiency by using a cotransfected Renilla expression plasmid, and values are represented as relative luciferase unit (RLU) (n = 4 duplicates, ± S.E., ** = p < 0.02).
COS7 cell extracts showed that the 4-amino acid 153–156 deletion reduced the interaction of LAP*/LAP with HDAC1 by 2–3-fold (Fig. 1E), verifying that this region is important for the binding of C/EBPβ to mSin3A/HDAC1. LAP*/LAP activate transcription of the C/ebp promoter in a manner that is restrained by their interaction with mSin3A/HDAC1 (5), and thus C/EBPβ153–156 would be predicted to be a more effective activator of C/ebp transcription than WT C/EBPβ. Indeed, C/EBPβ153–156 proved to be almost twice as effective as WT C/EBPβ in activating transcription from the C/ebp promoter in a transient transfection assay (Fig. 1F). Furthermore, although coexpression of additional exogenous HDAC1 together with WT C/EBPβ reduced the activation of C/ebp promoter transcription 2-fold, almost the level of the base line obtained with the empty pcDNA vector control, it had only a modest effect (0.25-fold) on the level of transcription induced by C/EBPβ153–156. These results were consistent with the reduced interaction of HDAC1 with C/EBPβ153–156 seen in coimmunoprecipitation experiments (Fig. 1E). Together these data provide strong evidence that amino acids 153–156 in C/EBPβ are required for its interaction with the mSin3A/HDAC1 corepressor complex.

C/EBPβ153–156 Induces Adipogenesis More Effectively than WT C/EBPβ—NIH 3T3 fibroblasts provide a model for direct analysis of the role of C/EBPβ in adipocyte differentiation. In the presence of exogenous C/EBPβ, the NIH 3T3 cells respond to an adipogenic mixture of MIX (an inhibitor of camp phosphodiesterases that elevate cAMP levels) and insulin to differentiate into lipid-accumulating cells that reproduce the characteristics of mature white adipocytes (15). Glucocorticoid treatment is not required for differentiation but potentiates the effect of MIX and insulin. By contrast, NIH 3T3 cells are completely refractory to the same mixture in the absence of exogenous C/EBPβ (15).

To enable us to focus on the beneficial effect of relieving HDAC1 inhibition of C/EBPβ in promoting differentiation, we titrated the MIX and insulin levels such that differentiation obtained in the presence of WT C/EBPβ was relatively low, as reflected by the levels of expression of the adipogenic marker adipin at day 4, and barely detectable Oil Red O staining of accumulated lipid at day 6 (Fig. 2A). Addition of the synthetic glucocorticoid dex markedly enhanced both adipin expression and Oil Red O staining, illustrating that the MIX/insulin treatment conditions were not limiting per se for differentiation.

When C/EBPβ153–156 was expressed in place of WT C/EBPβ, the efficiency of NIH 3T3 differentiation in response to insulin/MIX treatment increased, with adipin levels becoming readily detectable and Oil Red O staining being enhanced (Fig. 2A). When the differentiation mixture was supplemented with dex in addition to MIX and insulin, C/EBPβ153–156 continued to be more effective than WT C/EBPβ in inducing adipin expression and providing an incremental increase in Oil Red O staining. This result suggested that displacement of mSin3A/HDAC1 represents only one component of the total glucocorticoid effect on differentiation. Western analysis verified that C/EBPβ153–156 and WT C/EBPβ were expressed at the same levels in the cells (Fig. 2B).

Chromatin immunoprecipitation assays showed that both C/EBPβ153–156 and WT C/EBPβ were recruited to the C/eipa promoter with the same efficiency 24 h following insulin/MIX treatment (Fig. 2C). Furthermore, as reported previously in the NIH 3T3 cells (5, 6), HDAC1 recruitment to the promoter was strongly enhanced by the presence of WT C/EBPβ. In contrast, there was little or no increase in the recruitment of HDAC1 to the promoter in the presence of C/EBPβ153–156 compared with that observed following infection with the pLXSN control vector.

Quantitative RT-PCR performed on RNA extracted 24 and 48 h (Fig. 2D) following adipogenic stimulation showed that compared with WT C/EBPβ, C/EBPβ153–156 enhanced the induction of C/eipa mRNA, without significantly affecting the early expression of the adipocyte-specific Pparγ isoform, PPARγ2. This result is consistent with our previous observation that the interaction of mSin3A/HDAC1 with WT C/EBPβ specifically represses early C/eipa expression without affecting Pparγ expression as well as the results of other studies that indicate a primary role for C/EBPβ (which is expressed in NIH 3T3 cells following dex treatment) rather than C/EBPβ for PPARγ2 induction (5, 16, 17).

By contrast to the day 4 results, where overall differentiation in the presence of C/EBPβ153–156 was still enhanced by glucocorticoid, C/EBPβ153–156 was ineffective in further enhancing the early induction of C/eipa expression when glucocorticoids were included in the differentiation mixture. This result is consistent with the specific role for glucocorticoids in titrating mSin3A/HDAC1 from C/EBPβ and the C/eipa promoter at the onset of differentiation, which we have described previously (5, 6), and suggests that the additional effects of glucocorticoids observed in Fig. 2A are initiated subsequently and due to other targets of the steroid.

A second model that can be used to assess the role of C/EBPβ in differentiation is the 3T3 L1 preadipocyte cell line. Expression of C/EBPβ by retroviral infection is sufficient to drive differentiation of confluent 3T3 L1 cells in the absence of adipogenic mixture (15), as visualized by the presence of adipin 10 days post-confluence, as well as increased expression of C/eipa and PPARγ2 in the mature adipocyte cultures (Fig. 3, A and B). Oil Red O staining was similarly enhanced (Fig. 3C). Substitution of C/EBPβ153–156 for WT C/EBPβ resulted in a further enhancement of differentiation as reflected by adipin, C/eipa, and PPARγ2 expression and Oil Red O staining. These results reinforced the veracity of our finding that amino acids 153–156 in C/EBPβ play an important role in restraining its differentiation potential.

Active LIP Repressor Activity Is Mediated through Association with mSin3A/HDAC1—The importance of amino acids 153–156, which map to the N terminus of LIP, for HDAC1 association of C/EBPβ suggested the potential for LIP to act as an active transcriptional repressor. To determine the extent of HDAC1 interaction with LIP, full-length LIP, and LIPΔC, in which Pro-157 of LIP follows the initiator Met-152, were stably expressed in 3T3 L1 preadipocytes from a retroviral vector. Analysis of HDAC1 immunoprecipitates prepared from confluent cells revealed the specific association of LIP with HDAC1 at a 2.5 times higher level.
than observed for LIP6C (Fig. 4A). This establishes that LIP contains determinants sufficient for interaction with HDAC1 and that binding to LIP is reduced to the same extent by the same mutations that impact HDAC1 association with LAP/LAP*.

We next tested whether LIP could function as an active repressor of C/ebp/H9251 transcription in a transient transfection assay (Fig. 4B, upper panel). Here, increasing amounts of LIP or LIP6C were coexpressed in NIH 3T3 cells with fixed amounts of WT C/EBP/H9252 and a C/EBPα luciferase reporter construct. Increasing the level of LIP expression progressively reduced the activation of C/ebp/H9251 expression by LAP/LAP*. In contrast, LIP6C failed to affect transcription under these conditions, even when 100 ng of LIP expression vector was transfected, which provided for a ratio of LIP to LAP/LAP* in excess of 5 to 1 as assessed by Western analysis (Fig. 4C). This provides strong evidence that LIP functions as an active, HDAC1-dependent repressor of C/ebp/H9251 transcription and that in the absence of dex this activity is more important than heterodimerization for repression.

Treatment of the NIH 3T3 cells with dex enhanced the C/EBPβ/H9252-mediated activation of C/ebp/H9251 transcription by 4-fold (Fig. 4B, lower panel), as a result of the dissociation of HDAC1 from C/EBPβ and the resultant acetylation of the C/EBPβ and the C/ebp/H9251 promoter (5, 6). Nonetheless, LIP continued to efficiently repress C/ebp/H9251 transcription. Furthermore, LIP6C now also acted to repress C/ebp/H9251 transcription from this higher level albeit to a somewhat lesser extent than LIP. These results suggest that under conditions where the interaction of HDAC1 with C/EBPβ is reduced, passive repression by heterodimeriza-
tion of the higher level of C/ebpα transcription by LIP/LIP<sub>6C</sub> plays a predominant role in repression.

Abrogation of HDAC1 Association Compromises the Repression of Preadipocyte Differentiation by LIP—To assess the specific contribution of HDAC1 association to the repression of preadipocyte differentiation by LIP, we compared the ability of stably expressed LIP and LIP<sub>6C</sub> to repress the differentiation of 3T3 L1 cells in response to a strong adipogenic stimulus (insulin, MIX, and dex). Dex treatment was required for these experiments, as the low level of differentiation obtained by treating the cells with insulin and MIX alone was completely abrogated by both LIP and LIP<sub>6C</sub> (data not shown), making it difficult to determine a specific effect for mSin3A/HDAC1 association. Western blot analysis of the expression of adipsin, C/EBP<sub>α</sub>, PPAR<sub>γ</sub>, actin (B) and photomicrograph of Oil Red O staining of accumulated lipid (A magnification) (C) was performed at day 8 of differentiation.

FIGURE 3. C/EBPα<sub>153–156</sub> expression enhances differentiation of 3T3 L1 preadipocytes. A, Western blot analysis of stably expressed LAP, LAP<sup>*</sup>, and LIP levels expressed from C/EBP<sub>α</sub><sup>wt</sup> or C/EBPβ<sub>α</sub><sup>153–156</sup> retroviral vectors in 3T3 L1 cells 2 days following confluence. B and C, differentiation of retrovirally transduced 3T3 L1 cells expressing control (pLXSN) or C/EBP<sub>α</sub><sup>wt</sup> and C/EBPβ<sub>α</sub><sup>153–156</sup> Constructs and cultured in the absence of insulin, MIX, or dex. Western blot analysis of the expression of adipsin, C/EBP<sub>α</sub>, PPAR<sub>γ</sub>, actin (B) and photomicrograph of Oil Red O staining of accumulated lipid (A magnification) (C) was performed at day 8 of differentiation.

FIGURE 4. Mutation reducing LIP association with HDAC1 compromises LIP-mediated inhibition of C/epbα expression in the absence of dex. A, communoprecipitation assay with extracts of 3T3 L1 cells stably expressing WT LIP or LIP<sub>6C</sub> via retroviral transduction. Binding is compared with 5% of input, and levels of nonspecific (NS) binding were assessed using an antibody against Gal4 DNA-binding domain. Quantification of relative binding to HDAC1 is at the bottom of the panel and reflects three independent experiments (SEM, p < 0.05). B, analysis of C/EBPα promoter (<sup>−350/+7</sup>) activity in NIH 3T3 cells by a luciferase assay following transient transfection with 50 ng of WT C/EBPα expression vector together with the indicated amounts of LIP or LIP<sub>6C</sub> expression vectors. Cells were treated with vehicle (−DEX, top panel) or 1 μM dex (+DEX, bottom panel) for 16 h following transfection. RLU, relative luciferase unit. The results shown are quantified from three independent experiments performed in duplicate (SEM, p < 0.05). C, Western analysis showing the expression level of transiently transfected C/EBPα alone or cotransfected with 100 ng of either LIP or LIP<sub>6C</sub> in NIH 3T3 cells treated with either vehicle (ethanol) or dex.

levels of adipsin expression and prominent expression of C/EBPα and PPARγ2 in the mature adipocyte cultures (Fig. 5A). Oil Red O staining was similarly strong (data not shown).
When expressed from a retroviral vector, LIP and LIP$_{6C}$ expression persists throughout differentiation (Fig. 5A). However, endogenous LIP expression ceases within 3 days of the induction of differentiation, as C/EBPβ expression ceases and is replaced by C/EBPα (Fig. 5B) (15, 18). To exclude the possibility of an artifactual effect of the sustained expression of LIP in the differentiating preadipocytes, we repeated the 3T3 L1 differentiation experiments with cells in which LIP and LIP$_{6C}$ were expressed by transient transfection prior to the initiation of differentiation. Here, the levels of exogenous LIP were observed to be elevated over endogenous LIP at day 0 of differentiation (3 days post-transfection, Fig. 5C, left panel) but declined by day 2 of differentiation, such that exogenous LIP could no longer be detected over the endogenous factor (Fig. 5C, right panel). Under these conditions of temporally limited expression, LIP still effectively inhibited preadipocyte differentiation as reflected by a reduction in adipin, C/EBPα, and PPARγ2 at day 8 of differentiation (Fig. 5D). Indeed, efficient inhibition was observed following transfection of as little as 50 ng of LIP expression vector. By contrast, expression of LIP$_{6C}$ was substantially less effective in repressing differentiation, with transfection of 400 ng of LIP$_{6C}$ vector remaining less effective in inhibiting differentiation than 50 ng of LIP vector.

**HDAC1 Repression of C/ebpα Expression and Preadipocyte Differentiation Is Primarily Dependent on Its Association with LIP**—Although LAP/LAP* and LIP appeared to interact similarly with mSin3A/HDAC1, our experiments to this point did not comment on the extent to which mSin3A/HDAC1 activity depended on the individual C/EBPβ isoforms. Therefore, to study the extent to which HDAC1 could act through LAP/LAP* in the absence of LIP to blunt the activation of C/ebpα transcription and the stimulation of preadipocyte differentiation, we compared the effects of two mutants of C/EBPβ that express LAP/LAP*, but not LIP, and are distinguished by their ability to interact with HDAC1. C/EBPβ$_{M152A}$ is a C/EBPβ mutant in which the LIP translational initiation site was changed to Ala, thereby abrogating LIP expression, but in a manner that was not expected to affect HDAC1 association. In contrast, C/EBPβ$_{A151–156}$ contains a 6-amino acid deletion that eliminated the LAP/LAP* association site and also compromised HDAC1 association with LAP/LAP*. As verified by Western blots of NIH 3T3 cells (Fig. 6A) and coimmunoprecipitation from COS7 cell extracts (Fig. 6B), the A151–156 truncation eliminated LAP production and compromised LAP/LAP* association with HDAC1, whereas M152A eliminated LIP with little affect on HDAC1 association to LAP/LAP* (Fig. 6A and B). Similarly, C/EBPβ$_{M152A}$ retained the ability to recruit HDAC1 to the C/ebpα promoter in ChIP experiments, whereas C/EBPβ$_{A151–156}$ was as ineffective in recruiting HDAC1 (Fig. 6C) as we had shown earlier for C/EBPβ$_{A153–156}$ (see Fig. 2C).

To begin the assessment of how these two mutations affected the ability of C/EBPβ to activate transcription of C/ebpα, the two mutants and WT C/EBPβ were transiently transfected with a C/EBPα reporter in NIH 3T3 cells (Fig. 6D). As expected, C/EBPβ$_{A151–156}$ enhanced C/ebpα transcription more efficiently than WT C/EBPβ, to the same extent observed earlier with C/EBPβ$_{A153–156}$ (see Fig. 1E).

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**Active Repression by LIP Mediated through HDAC1**

Expression of LIP from the same viral vector reduced adipin expression below the threshold of detection, and reduced C/EBPα and PPARγ2 expression at day 8. Oil Red O staining was similarly reduced (data not shown). By contrast, expression of LIP$_{6C}$ had an intermediate effect on differentiation, reducing adipin expression much less than LIP and leaving day 8 C/EBPα and PPARγ2 expression elevated. Thus, even in an environment where HDAC1 titration from LAP/LAP* was stimulated by dex, the early interaction of mSin3A/HDAC1 with LIP still appears to impart a substantial component of the repressive potential of LIP.

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**FIGURE 5. Reducing mSin3A/HDAC1 binding compromises the ability of LIP to repress preadipocyte differentiation.** A, 3T3 L1 cells retrovirally transduced to express either pLXSN vector, LIP$_{6C}$ or LIP$_{6C}$ were differentiated for 8 days following MID treatment. A Western blot analysis was performed to assess adipin, C/EBPα, PPARγ, and actin levels. The reduced level of actin expression in the pLXSN lanes reflects a 3-fold decrease in the amount of extract used, which was required to prevent overexposure of the pLXSN adipin track on the blot. B, time course Western analysis of endogenous C/EBPβ levels in 3T3 L1 cells following their treatment with either M1 (−) or MID (+) at 2 days post-confluence. C, Western blot analysis showing the expression level of endogenous (pcDNA mock plasmid) and exogenous LIP in 3T3 L1 cells 3 days following transfection with 100 ng of the DNA constructs (left panel). The transfected 3T3 L1 cells were also harvested 2 days post-treatment (or 5 days following transfection with the LAP constructs) to evidence a decrease in exogenous LIP expression, as shown by similar expression levels in all three lanes (right panel). D, 1 day before confluency, 3T3 L1 cells were transiently transfected with increasing amount of the pcDNA-LIP or pcDNA-LIP$_{6C}$ constructs. Three days later, the cells were induced to differentiate with MID for 8 days and harvested for immunoblot analysis as in A, except in this instance loading was equal in all lanes.

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however, C/EBPβM152A closely mimicked C/EBPβΔ151–156 in its transcriptional activation potential, even though it retained WT ability to interact with HDAC1. This suggested, at least under the conditions we employed, that the effect of HDAC1 in repressing the activation potential of C/EBPβ was minimal in the absence of LIP.

To determine whether this result was the product of artificial transient transfection conditions, we proceeded to compare the effects of C/EBPβΔ151–156 and C/EBPβM152A on the differentiation of NIH 3T3 cells when stably expressed at physiological levels from a viral vector (Fig. 7). WT C/EBPβ, C/EBPβΔ151–156, and C/EBPβM152A expressed to similar levels, with LIP expression only detected from the WT C/EBPβ (Fig. 7A). Again, when compared with the WT C/EBPβ, both mutants behaved similarly, strongly enhancing differentiation as reflected by very similar severalfold enhancements of adipin accumulation (Fig. 7B) and a concomitant enhancement of Oil Red O staining (data not shown). Although overall differentiation was also strongly enhanced by dex treatment, C/EBPβΔ151–156 and C/EBPβM152A again provided for a similar enhancement of differentiation above the level obtained with C/EBPβ, reinforcing that the interaction of HDAC1 with LAP/LAP* seemed insufficient to significantly repress differentiation.

Quantitative RT-PCR analysis of the onset of C/ebpa and Pparγ2 expression at 24 h reiterated that C/EBPβM152A and C/EBPβΔ151–156 enhanced the early expression of C/ebpa similarly in both the absence and presence of dex (Fig. 7C), again consistent with our earlier result with C/EBPβΔ153–156 (Fig. 2D). Thus, it appears that although LAP/LAP* readily inter-

**FIGURE 6. Repressive effect of mSin3A/HDAC1 association with C/EBPβ on C/ebpα expression is mediated primarily through LIP association.** A, Western analysis of the expression levels of exogenous WT C/EBPβ, C/EBPβM152A, and C/EBPβΔ151–156 in NIH 3T3 cells expressed by a transient transfection. B, communoprecipitation analysis of the binding of FLAG-HDAC1 to C/EBPβwt, C/EBPβM152A, and C/EBPβΔ151–156. Whole cell lysates prepared from transiently transfected COS7 cells were immunoprecipitated (IP) with a FLAG affinity resin. C, chromatin immunoprecipitation analysis of HDAC1 association with the C/ebpα promoter in NIH 3T3 cells stably expressing retroviral C/EBPβ constructs. Cells were treated with MI for 24 h prior to analysis. A representative result from three independent experiments is shown. D, transient transfection analysis of the activation of C/ebpα-luciferase expression by C/EBPβ, C/EBPβM152A, and C/EBPβΔ151–156 in NIH 3T3 cells. Cells were treated with either vehicle ( ) or 1 μM dex ( ) for 16 h following transfection. Numbers represent the averages of four independent experiments performed in duplicate (± S.E., * = p < 0.05).

**FIGURE 7. Repressive effect of mSin3A/HDAC1 association with C/EBPβ on preadipocyte differentiation is mediated primarily through LIP association.** A, Western blot analysis of stably expressed C/EBPβwt, C/EBPβM152A, and C/EBPβΔ151–156 in NIH 3T3 cells just prior to the initiation of differentiation (day 0). B, Western analysis of adipin and actin in cells harvested 4 days post-induction in the absence (MI) or presence (MID) of 250 nM dex. C, real time RT-PCR analysis of C/ebpα (upper panel) and Pparγ2 (lower panel) mRNA expression at 24 h following the induction of differentiation with MI ( ) or MID ( ). mRNA levels were normalized to actin (n = 3, ± S.D., * = p < 0.05 and ** = p < 0.01). Quantification of both C/EBPβ and PPARγ2 are relative to MID βwt set as 1 relative unit.
However, in contrast to our earlier result, where C/EBPβ<sub>153–156</sub> provided for the same enhancement of Ppar expression as WT C/EBPβ, in this instance both C/EBPβ<sub>M152A</sub> and C/EBPβ<sub>151–156</sub> also resulted in an increase in Ppar expression above the level obtained with WT C/EBPβ. Together, these results strongly reinforce support for the hypothesis that Ppar expression is repressed by LIP in an HDAC1-independent manner.

Overall, the results of this study significantly enhance our understanding of the means through which HDAC1 represses preadipocyte differentiation and the activation of C/ebpα expression through its interaction with C/EBPβ. Most notably, although HDAC1 appears to interact similarly with LAP/LAP* and LIP, its repressive effects appeared to depend only on the presence of LIP.

**DISCUSSION**

In this study, we have delimited key determinants for the interaction of mSin3A/HDAC1 with the LAP*, LAP, and LIP isoforms of C/EBPβ and described how the mSin3A/HDAC1 interaction functions as a regulatory switch in controlling the transcriptional activation potential of C/EBPβ in preadipocytes and fibroblasts stimulated to differentiate into mature adipocytes. Interestingly, our results showed that although mSin3A/HDAC1 exhibited a similar ability to interact with LAP/LAP* dimers, LAP/LAP*-LIP heterodimers, and LIP homodimers, its repression of C/ebpα transcription and preadipocyte differentiation appeared to depend exclusively on the presence of LIP. These results provide the first evidence for LIP as an active transcriptional repressor. When combined with our previous studies describing the glucocorticoid-mediated control of mSin3A/HDAC1 binding to C/EBPβ (5, 6), our results reveal a mechanism for the stepwise control of C/EBPβ transcriptional regulatory activity that allows it to range from a strong activator to a strong repressor of transcription.

White adipose tissue is an important metabolic organ that functions to maintain energy homeostasis by storing excess energy in the form of triglycerides and lipid. Unlike many organs, white adipose tissue has an almost unlimited ability to grow to accommodate the storage need. However, mature adipocytes do not replicate. Thus, tissue expansion is determined by creation of new adipocytes through differentiation from a proliferating pool of preadipocytes. Once created, adipocytes are long lived, and weight loss is not accompanied by a substantial reduction in adipocyte number. Therefore, during times of nutrient excess it is particularly important that the body has an effective means to regulate the creation of new adipocytes so that the number of cells created balance the storage need.

Previous studies have shown that in preadipocytes stimulated with insulin and MIX, mSin3A/HDAC1 and p300/GCN5 interacts with C/EBPβ in a manner that leads to competition between the two factors for acetylation of the C/ebpα promoter and the acetylation of LAP/LAP* between amino acids 98 and 102. The addition of glucocorticoids to the stimulatory mixture promotes HDAC1 degradation over the first 24 h of preadipocyte differentiation. This enables the accumulation of GCN5-mediated acetylation of lysines 98–102 of LAP/LAP* that interferes with continued mSin3A binding (5, 6).

In this study, we have determined that mSin3A/HDAC1 interacts similarly with the three C/EBPβ isoforms and that this binding can be crippled by a 4-amino acid deletion (Δ153–156) that is in the middle of LAP/LAP* and at the N terminus of LIP. This mutation decreased mSin3A/HDAC1 binding and recruitment to the C/ebpα promoter and provided for a concomitant increase in the induction of C/ebpα transcription and preadipocyte differentiation. Our initial results suggested that mSin3A/HDAC1 binding to C/EBPβ depended on the ability of C/EBPβ to form dimers. However, the dimer composition did not seem to have any appreciable effect on binding.

Predictably, increasing the expression level of LIP compared with LAP/LAP* led to a decrease in the level of C/ebpα promoter activity. However, in contrast to previous studies that have concluded that LIP repression is mediated passively through heterodimerization with LAP/LAP* (thereby reducing the number of activation domains recruited to C/EBPβ regulatory elements) and the competition of LIP homodimers for binding to DNA regulatory motifs, our results show that repression of transcription by LIP is first dependent on active repression through the recruitment of mSin3A/HDAC1. Thus, mutations that abrogated mSin3A/HDAC1 binding to LIP (LIP<sub>6C</sub>) relieved LIP-dependent transcriptional repression. Upon glucocorticoid treatment, which enhances C/EBPβ transcriptional activity through titration of HDAC1, active repression by LIP was reduced and passive repression was revealed, as a repressive contribution emerged for LIP<sub>6C</sub>. Although these results do not fundamentally change our perspective for transcriptional repression by LIP in the presence of LAP/LAP*, it does indicate that repression could be manipulated by agents that affect HDAC1 deacetylase activity. It also suggests that recruitment of LIP to transcriptional regulatory elements in the absence of LAP/LAP* would not simply be neutral for transcription but would be expected to repress the positive effects of surrounding transcriptional activators.

Although mSin3A/HDAC1 binding appears to be important for the repression of C/ebpα expression, the mutations that disrupted mSin3A/HDAC1 binding did not affect Ppar expression under the same conditions. In the first instance, these results are consistent with earlier reports that glucocorticoid treatment and mutations that disrupt mSin3A binding by mimicking LAP/LAP* acetylation also failed to have an impact on Ppar expression (5, 6). In contrast, abrogating LIP expression through the M152A mutation of its translational initiation site affected the induction of C/ebpα and Ppar transcription equally. An exact mechanism is difficult to predict on the basis of these initial results. However, one possibility is that the active HDAC1-dependent repressor activity of LIP is promoter-specific and thus is not relevant to the Ppar promoter. Alternatively, as Ppar expression appears to be primarily dependent on C/EBPβ rather than C/EBPβ (5, 16, 17), it may be that LIP is only able to influence C/EBPβ activity through a passive mechanism. In any event, more experimentation will be needed to resolve this interesting observation.

Elimination of LIP expression allowed us to confirm that mSin3A/HDAC1 interacted with LAP/LAP* independently from the presence of LIP, while still showing the increase in C/ebpα transcription and induction of preadipocyte differen-
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FIGURE 8. Schematic presentation of the interplay of p300/GCN5 and mSin3A/HDAC1 interacting with C/EBPα at the C/EBPα promoter. Molecular complex assembly nucleated by C/EBPβ isoforms and dimer combinations at the C/EBPα promoter in preadipocytes stimulated to differentiate with insulin and MIX in the absence (left) or presence of dexamethasone (right). LAP/LAP* (represented simply here as LAP), LIP, p300/GCN5, mSin3A, and HDAC1 are represented as indicated. Ac = acetylation at amino acids 98–102 in LAP/LAP* that interferes with mSin3A binding to C/EBPα (6). The relative levels of transcription are reflected by the sizes of arrows. The dotted line representing HDAC1 in the lower right panel reflects the decrease in HDAC1 levels that occurs in response to dexamethasone, which would be expected to reduce the recruitment of HDAC1 to the promoter.

Unexpectedly, however, extending the LAP/LAP* mutation to compromise mSin3A/HDAC1 association failed to provide the additional benefit expected for C/ebpα expression and differentiation, at least under the conditions tested. LAP/LAP* dimers bring together two transcriptional activation domains that would be expected to double the loading of p300/GCN5 (which bind to monomeric activation domains) compared with LAP/LAP*-LIP heterodimers (6).3 Thus, at first glance it may be possible that increased coactivator loading on LAP/LAP* is able to overcome the repressive presence of mSin3A/HDAC1.

Although our work begins to detail the interplay of LAP/LAP*, LIP, mSin3A/HDAC1, and the transcriptional coactivators such as p300/GCN5 that provides for a fine-tuning of the transcriptional regulatory potential of C/EBPβ, it is clear that more detailed studies are needed to fully resolve the mechanisms at play. Nonetheless, Fig. 8 makes an early attempt to rationalize the regulatory events observed schematically.

In all of the cell types employed in this study, the ratio of LAP/LAP* to LIP expression was ~1:1. This suggests the predominant form of C/EBPβ to be heterodimers of LAP/LAP* and LIP, with LAP/LAP* and LIP homodimers occurring equally but at lower levels (2:1:1). LAP/LAP*-LIP heterodimers, with a single transcriptional activation domain, would exhibit similar loading of mSin3A/HDAC1 and p300/GCN5, conditions under which the effect of the mSin3A/HDAC1 repressive activity would be evident (Fig. 8, top panel). Here, disruption of mSin3A/HDAC1 binding, as a result of glucocorticoid treatment, results in an increase in activation. By contrast, the presence of a second activation domain in LAP/LAP* dimers would favor p300/GCN5 activation activity over mSin3A/HDAC1-mediated repression and would result in elevated base-line transcriptional activation that would benefit minimally from additional stimulation via glucocorticoid treatment (Fig. 8, middle panel). LIP homodimers would interact exclusively with mSin3A/HDAC1 (Fig. 8, bottom panel). In this instance, active repression of transcription would be expected to be relieved by mutations that disrupt mSin3A binding, although passive repression would continue.

Studies of preadipocytes have shown that commitment to differentiation occurs stochastically within cultures, with increasing strength of adipogenic stimulus leading to the commitment of increasing numbers of preadipocytes to differentiation. Molecular studies have shown that C/EBPβ functions directly prior to the onset of commitment, to initiate the transcription of the commitment factors C/ebpα and Pparγ. Once activated beyond a certain threshold, autocrine and cross-regulation of C/ebpα and Pparγ drive the cells to terminal differentiation. Our results provide a means through which C/EBPβ activity can be closely regulated so as to determine the extent of stochastic commitment. Under mild adipogenic conditions or when glucocorticoid levels are low, the reduced potency of C/EBPβ would limit the number of cells that could attain the threshold of C/ebpα expression required for commitment. By contrast, in response to strong stimuli, including elevated steroid levels, a stronger, broader response of C/ebpα would be anticipated. Similarly the ratio of LAP to LIP expression, which can be manipulated under some circumstances (19–22), would also control the ability of the cell to reach the commitment threshold of C/ebpα expression.

One open question is whether HDAC1 contributes an essential deacetylase activity to the repression of C/EBPβ or whether it functions only as a structural component of a larger complex that includes other HDACs. Our recent results support a direct enzymatic role for HDAC1, in the regulation of C/EBPβ activity as HDAC1 mutants lacking histone deacetylase activity were unable to repress C/EBPβ-dependent C/ebpα transcription or preadipocyte differentiation.4

Finally, regulation of mSin3A/HDAC1-p300/GCN5 equilibrium on C/EBPβ is only one of several means through which C/EBPβ activity is known to be controlled. Thus, C/EBPβ is acetylated at additional sites and is also regulated by a variety of post-translational modifications that affect its DNA binding ability, stability, and association with cofactors (23–29). Layering of these regulatory interactions into the interplay of mSin3A/HDAC1 and p300/GCN5 with LAP/LIP may be expected to further enhance the regulation of C/EBPβ transcriptional activity, positioning C/EBPβ as a precisely tunable transcriptional rheostat for the activation of the factors that commit preadipocytes to differentiation. It may also be expected that the fine-tuning of C/EBPβ transcriptional regu-

3 H.-S. Abdou and R. J. G. Haché, unpublished observations.

4 C. Kuzmochka, E. Atlas, and R. J. G. Haché, manuscript in preparation.
latory potential will play equally important roles in guiding the differentiation of other tissues and cell types (liver, uterus etc) in which C/EBP plays a prominent role.

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