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FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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REGULATION OF C/EBPβ TRANSCRIPTION AND PREADIPOCYTE DIFFERENTIATION BY HISTONE DEACETYLASE 1 AND GCN5.

by

Nadine Louise Wiper

Graduate Program
in
Biochemistry

A thesis submitted in partial fullfillment
of the requirements for the degree of
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DEDICATION

I dedicate this thesis to my parents, Margo and Jim Wiper. My parents made the long days and nights in the lab, the papers to write, the scholarships to earn and the general hardship of graduate school bearable. I cannot count how many times they helped me get through rough times with encouraging words, with a home-cooked meal, babysitting, or even a new pair of lab worthy shoes!

My parents also provided me with the non-tangibles required to survive graduate school. My father gave me an insatiable curiosity, a desire to know how and why, that made scientific research incredibly satisfying. However, curiosity is "necessary but not sufficient" to complete the task. My mother gave me the tenacity to push forward and through the rougher times.

I wish to also acknowledge my husband Christian, who has seen so little of me over the past 5 years. His support and understanding of my commitment to this project is greatly appreciated.

To my daughter Sophie, for allowing me to finish writing this thesis before being born, if only by a few hours!

How lucky I have been to have all the support I do.

This thesis is a team effort. Thank you all for your individual contributions.

Much love.

ABSTRACT

Previous studies in this laboratory demonstrated that the glucocorticoid receptor (GR) ligand binding domain (LBD) can enhance transcription mediated by the CCAAT/enhancer binding protein C/EBPβ. This result suggests that the receptor can act in a non-classical manner to positively affect transcription without directly binding DNA.

To study the physiological impact of the potentiation of C/EBPβ activity by the GR LBD, we focused on preadipocyte differentiation as both GR and C/EBPβ influence early events. Using retroviral expression of the GR LBD in 3T3 L1 cells, a 3-fold increase in preadipocyte differentiation was observed when cultures were induced to differentiate in a cocktail containing dexamethasone. The GR LBD was also able to enhance transcription mediated by DNA-bound C/EBPβ from the C/EBPα promoter and its expression increased C/EBPα protein levels during preadipocyte differentiation. The effect of the GR LBD was accomplished through the targeted degradation of a sub-population of the transcriptional repressor histone deacetylase 1 (HDAC1), which associates with C/EBPβ via corepressor protein mSin3A. In the absence of glucocorticoids, HDAC1 maintains the C/EBPα promoter in a deacetylated state. The addition of steroid to the differentiation cocktail causes the degradation of the HDAC1 found in the C/EBPβ-associated complex, a release of mSin3A, increased acetylation of the promoter histone H4 and recruitment of RNA polymerase II leading to maximal transcription from the C/EBPα promoter.

C/EBPβ itself is acetylated by the histone acetyltransferases GCN5 and PCAF within lysines 98, 101 and 102. In 3T3 L1 preadipocytes where only GCN5 is expressed, GCN5 coprecipitates with C/EBPβ and can be found at the C/EBPα promoter by chromatin immunoprecipitation. The acetylation of C/EBPβ is essential for development of full transcriptional activation potential and for differentiation of preadipocytes. Mutation of these

residues leads to loss of transcriptional potentiation by GR and a striking decrease in the number of differentiated cells in both NIH 3T3 and 3T3 L1 cultures. Following glucocorticoid treatment, the mutant C/EBPβ remains associated with HDAC1 and is inefficient in driving C/EBPα transcription. Acetylation may be a required modification to prevent C/EBPβ from repression by HDAC1.

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Thank you.

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INTRODUCTION

Nuclear Receptors

Nuclear receptors comprise a large family of transcription factors that are present in both vertebrates and invertebrates (Mangelsdorf et al., 1995). The family is defined by the presence of two highly conserved domains: a centrally located DNA-binding domain, and a C-terminal ligand binding domain. In fact, nuclear receptors share a modular protein structure that includes an N-terminal activation function, termed AF-1, a ligand-dependent C-terminal activation function (AF-2), and a flexible hinge region nestled between the DNA-binding domain and the ligand binding domain. Despite the presence of a ligand binding domain, not all nuclear receptors identified to date have known ligands. These receptors for whom no ligand has been identified are therefore called "orphan" receptors (Mangelsdorf and Evans, 1995).

Until recently, the nuclear hormone receptor superfamily was subdivided into 4 classes, based on DNA-binding and dimerization properties (Mangelsdorf et al., 1995). Class I nuclear receptors are comprised of the steroid hormone receptors, which include the glucocorticoid receptor (GR), the mineralocorticoid receptor as well as the androgen, estrogen and progesterone receptors, and were grouped together due to their binding to an inverted palindromic DNA motif as homodimers (Strahle et al., 1987). The class II nuclear receptors, such as the thyroid hormone receptor, the retinoic acid receptor and the peroxisome proliferator activated receptors (PPARs) which heterodimerize with the promiscuous binding partner retinoic acid X receptor (RXR), bind DNA sites that consist of direct repeats (Leid et al., 1992; Naar et al., 1991; Umesono et al., 1991). The rest of the receptors, consisting mostly of orphan receptors were further classified based on their dimeric or monomeric association with DNA elements (Laudet and Adelmant, 1995; Ueda et al., 1992; Wilson et al., 1991; Wilson et al., 1993). The Class III receptors include RXR and HNF-4 and bind to DNA as homodimers to response elements formed by direct repeats

of the consensus sequence. The Class IV receptors bind to DNA as monomers and include NGFI-B and ELP/SF-1.

Recently, the nuclear hormone receptor superfamily, now comprising 65 members, was reclassified based on the evolution of the DNA-binding and the ligand binding domains (Nuclear Receptors Committee, 1999). The superfamily is now subdivided into 6 subclasses in which members of the same group share 80-90% homology within the DNA-binding domain and at least 40% homology within the ligand-binding domain. Subfamily 1 includes 11 groups of receptors including the retinoic acid receptor, the thyroid receptor and the PPARs as well as the vitamin D receptor. Subfamily 2 contains 6 groups of receptors including HNF-4 and RXR, both of which can bind to directly repeated DNA motifs as homodimers. The third subfamily includes three groups and houses the estrogen receptor, the orphan estrogen receptor related receptors and the steroid receptors including GR. Subfamily 4 includes only one group of genes related to NGFIB. The fifth subfamily contains the Drosophila FTZ-F1 receptor, its vertebrate homologues and the DHR39 receptor. The last subfamily has only one gene, the GCNF1 receptor. Nuclear receptors that lack one of the conserved domains, either the DNA-binding domain or the ligand binding domain are grouped together in subfamily 0.

The Glucocorticoid Receptor

GR was the first cloned receptor of the nuclear hormone superfamily and since then has been studied extensively as the prototypical steroid hormone receptor (Hollenberg et al., 1985; Miesfeld et al., 1986). GR is found in all nucleated cells and transduces the effects of glucocorticoids *in vivo*. Two forms of the human glucocorticoid receptor were cloned initially and designated GRα and GRβ (Hollenberg et al., 1985). GRα is the major form and represents the full-length receptor that binds its hormone cortisol with an affinity of 11-14 nM and the synthetic glucocorticoid dexamethasone (dex) with an affinity of 3.7-4.6 nM (Hellal-Levy et al.,

1999; Ray et al., 1999). The second isoform GRβ is truncated at the C-terminus within the ligand binding domain and is therefore not able to bind cortisol (Oakley et al., 1996). Initially believed to be an artifact of the cloning technique, GRβ is now regarded as an alternative splicing product which may have a specific role in the modulation of transcriptional responses by the full-length receptor (Encio and Detera-Wadleigh, 1991). This isoform appears to be constitutively localized to the nucleus of the cell, and via dimerization with GRα may act to repress transcriptional activation by the full-length receptor (Bamberger et al., 1995; Oakley et al., 1997).

Structure

The rat GR, cloned in 1986, spans 795 amino acids and has a molecular weight of 97 kDa by SDS-PAGE (Miesfeld et al., 1986). The rat GR shares 83% identity with the human receptor. As with all nuclear receptors, the centrally located DNA-binding domain, consisting of two zinc fingers (2 cys 4), spans 66 amino acids and is located from amino acids 440-505 (Berg, 1989). The first zinc finger contains the "P-box" and is responsible for sequence specific interactions with the DNA palindrome. The second finger is responsible for DNA backbone contacts and for DNA-dependent dimerization of the receptor through the "D-loop" (Luisi et al., 1991). The ligand binding domain (amino acids 547-795) is made up of 11 α-helices which take on a three-layer helical sandwich shape characteristic of the nuclear receptor superfamily (Bledsoe et al., 2002; Wurtz et al., 1996). Nestled between the DNA and ligand binding domains is a flexible hinge region. The hinge not only provides the necessary flexibility required for dimerization dependent DNA binding but also contains some motifs necessary for proper functioning of the receptor.

Binding of heat shock proteins (hsp90, hsp70, hsp56) and immunophilins to the ligand binding domain, required for efficient hormone binding, masks a nuclear localization signal with the core lysine residues at amino acids 513-515 within the hinge of the receptor (Picard and

Yamamoto, 1987; Pratt and Toft, 1997). Thus, with the nuclear localization signal covered, the naïve GR remains localized in the cytoplasm (Pratt, 1993). After diffusion of glucocorticoids through the cell's plasma membrane, the ligand binding domain binds the hormone, which induces a conformational change in the last helix of the receptor (Moras and Gronemeyer, 1998). This helix, which houses the AF-2, adopts a new position which stabilizes the receptor in an active conformation thereby allowing interaction with coactivating accessory molecules (Danielian et al., 1992; Moras and Gronemeyer, 1998). The new conformation also causes the shedding of the heat shock proteins and immunophilins, revealing the nuclear localization signal and allowing efficient translocation of the receptor into the nuclear compartment of the cell where it can influence the transcription of steroid responsive genes (Guiochon-Mantel et al., 1992). Cooperative binding of the receptor to DNA requires dimerization within the DNA-binding domain (Dahlman-Wright et al., 1991; Eriksson and Wrange, 1990). However, recent work has demonstrated that determinants within the hinge region (amino acids 511-539) form a solution dimerization interface that permits association of GR monomers before translocation into the nuclear compartment, and prior to DNA-contact, and was verified by crystal structure analysis of the LBD (Bledsoe et al., 2002; Savory et al., 2001).

Physiological functions

Ablation of GR in knockout mice highlights the importance of GR function *in vivo*. The vast majority of mice with homozygous loss of GR ($GR^{-/-}$) die within hours of birth due to pulmonary atelectasis and respiratory failure (Cole et al., 1995). As well, these mice do not express liver enzymes involved in gluconeogenesis and have severely deregulated glucose homeostasis. The adrenal glands of these animals lack the medulla region and do not produce adrenaline. Defects in this organ suggest that glucocorticoid signaling is required for the proliferation and survival of adrenal medulla cells. The surviving homozygotes show increased

levels of circulating adrenocorticotropic hormone (ACTH) and corticosterone (the endogenous glucocorticoid in rodents) due to deregulation of the hypothalamic-pituitary-adrenal axis negative feedback loop. Heterozygotes ($GR^{-/+}$) also demonstrate higher than normal levels of ACTH and corticosterone, with levels that fall between wild type and null mice. These results suggest that responses to corticosterone are affected by gene dosage and that precise levels of GR are required for strict control of its effects.

The physiologocical response to cortisol involves many different systems. Glucocorticoids regulate glucose homeostasis in times of stress by stimulating protein catabolism and by promoting gluconeogenesis (Granner and Pilkis, 1990; Pilkis and Granner, 1992). Glucocorticoids released by the adrenal cortex also cause vasoconstriction and are a powerful anti-inflammatory agents (Whitworth et al., 2000). This last effect is the basis for the extensive therapeutic use of glucocorticoids.

Apart from maintaining homeostasis in the adult, glucocorticoids also play an important role in the development and differentiation of the organism. A role for cortisol has been identified in lung maturation, in the turnover of bone, during stress erythropoiesis, during the formation of long-term memory (long term potentiation) and in the differentiation of fibroblast precursors into white adipose tissue (Bauer et al., 1999; Gregoire et al., 1998; Hirayama et al., 2002; McGaugh and Roozendaal, 2002; Roozendaal, 2000; Smith and Sabry, 1983; Torday, 1980).

These effects of glucocorticoids, mediated by GR, are the result of altered transcription of steroid-responsive genes. The receptor, which can both activate and repress transcription in a ligand-dependent manner, modifies transcriptional responses in different ways depending on the presence and configuration of response elements and protein-protein interactions. Thus, the receptor's action can vary with cell type and can be modified by signals that converge at hormone responsive genes.

Modes of glucocorticoid action

To date, GR has been shown to influence transcription in two major ways: through genomic and non-genomic actions. The receptor can bind to DNA directly and act to enhance transcription from steroid responsive promoters. DNA-binding by GR can also result in the repression of transcription through a direct mechanism or by interfering with the actions of other transcription factors. These collectively are the genomic effects of GR (Fig. 1A). The receptor can also act to influence transcription through non-genomic mechanisms, which rely on protein-protein interactions to modulate the transcriptional response (Fig. 1B).

Genomic mechanisms

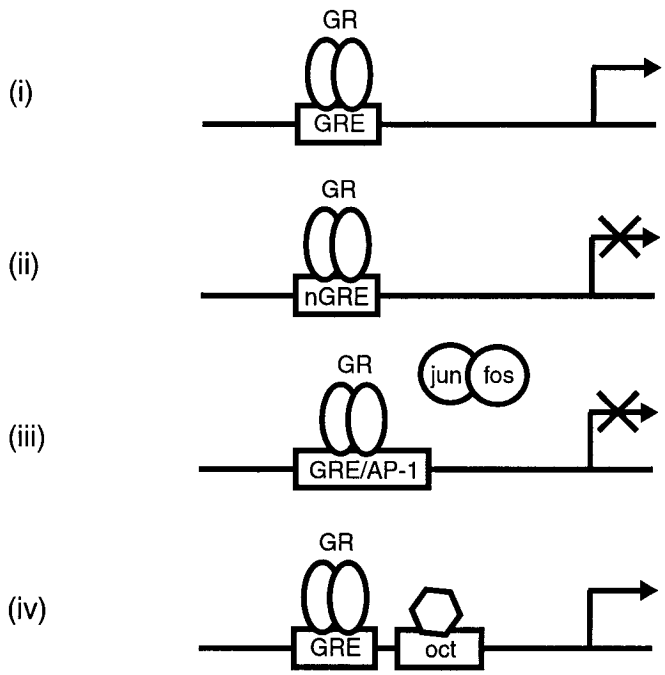
The liganded GR can bind to specific DNA motifs within the promoters of steroid-responsive genes called glucocorticoid response elements, or GREs. The GRE consists of an inverted hexameric DNA sequence repeat separated by three non-conserved nucleotides, with the consensus sequence GGTACAnnnTGTTCT (Funder, 1996). In fact, this consensus sequence is recognized by all of the steroid receptors, with the exception of the estrogen receptor, due to their highly conserved DNA-binding domains (Beato et al., 1995). Cooperative homodimerization of the receptor creates higher affinity binding of GR to the DNA element (Drouin et al., 1989). DNA binding of the homodimer promotes the activation of transcription, likely through the interaction of the receptor with coregulatory molecules, which possess the ability to open up chromatin by acetylation and ATP-dependent mechanisms, and through the attraction of basal transcription factors (Fig. 1Ai). It has long been thought that mutation of the D-loop of the DNA binding domain of GR results in a loss of DNA binding by the receptor and therefore loss of transcriptional activation from a GRE. Recent investigations by Adams and coworkers has presented evidence that such dimerization mutants of the receptor may in fact be able to bind DNA as monomers and can activate transcription from the phenylethanolamine

Figure 1. The glucocorticoid receptor can influence transcription through genomic and non-genomic actions.

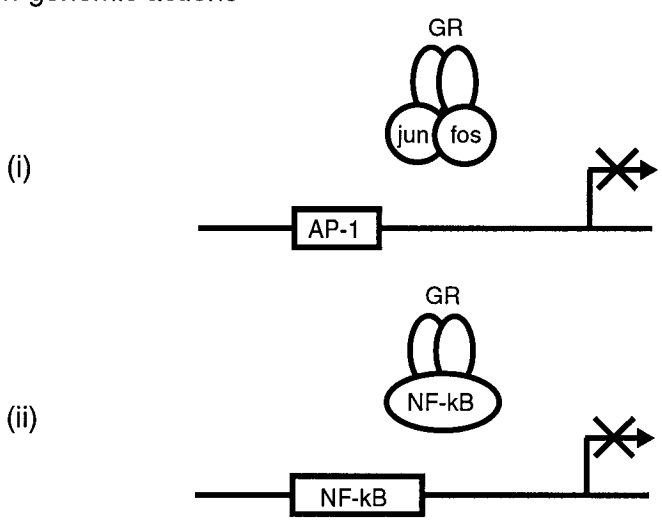
(A) Direct contact between the receptor and a glucocorticoid response element (GRE) can lead to enhanced transcription of a target gene (i). Association of the receptor with a negative response element (nGRE) leads to repression of the transcriptional response (ii). Binding of the receptor to a composite response element, where the DNA binding sites for both GR and the transcription factor AP-1 partially overlap, prevents the binding of the positive acting AP-1 dimer. Under these conditions, promoter activity is reduced when GR binds the DNA motif (iii). Association of the glucocorticoid receptor with oct transcription factors results in a potentiation of transcription. The protein-protein interaction between the receptor and oct increases the local concentration of oct at promoter which have both GREs and an oct response element such as the mouse mammary tumour virus long terminal repeat (iv).

(B) Protein-protein interaction between the glucocorticoid receptor and the AP-1 transcription factor results in decreased binding of AP-1 to its response element and a repression of transcription from AP-1 promoters (i). Similarly, when the glucocorticoid receptor interacts with transcription factor NF-kB, the association prevents binding to the NF-kB response element and the subsequent activation of transcription (ii)

A Genomic actions



B Non-genomic actions



N-methyltransferase promoter (Adams et al., 2003). Also challenging the notion of cooperative DNA binding is the observation that the glucocorticoid receptor can in fact dimerize through an interface located in the hinge region of the receptor. This interface allows interaction of the receptor in the absence of DNA binding (Bledsoe et al., 2002; Savory et al., 2001).

On some promoters, such as the keratin genes or the proopiomelanocortin gene, the binding of liganded GR to the GRE results in the repression of transcription (Fig. 1Aii) (Drouin et al., 1993; Radoja et al., 2000). The transcriptional repression occurs through atypical GREs known as negative GREs. The mechanism of repression is unknown under these circumstances, but it appears likely to involve an alternative interaction of GR with DNA. In fact, evidence exists suggesting that negative GREs direct GR to bind to DNA as monomers, or as complexes of homodimer and monomer (Drouin et al., 1993; Radoja et al., 2000).

The interference or crosstalk that occurs between the glucocorticoid receptor and other transcription factors has also been extensively studied. In the context of certain promoters, such as the human collagenase I gene, the response elements for GR and the dimeric transcription factor AP-1 partially overlap, forming a composite response element (Yang-Yen et al., 1990). The binding of GR to the composite element effectively excludes DNA-binding by AP-1, thereby preventing the activation of transcription (Fig. 1Aiii). The close proximity of response elements can also lead to synergistic activation of transcription. In the case of transcription from the mouse mammary tumour virus promoter, the presence of liganded GR and the octamer transcription factor Oct-1 or Oct-2 results in much greater transcription than that observed by each factor individually (Prefontaine et al., 1998). It is hypothesized that protein-protein interactions between the liganded GR and Oct-1 brings Oct-1 into close proximity to its response element in the promoter when GR binds DNA (Fig. 1Aiv). This would effectively increase the local concentration of Oct-1 and enhance its DNA binding and transcriptional potential.

Non-genomic mechanisms

GR can also influence transcription through non-genomic mechanisms (Fig 1B). Liganded GR has been shown to interfere with AP-1-mediated transcription from promoters that lack glucocorticoid response elements (Fig. 1Bi) (Schule et al., 1990). Although the exact mechanism at work is unknown, it is clear that the protein-protein interaction between AP-1 and GR is necessary for the crosstalk to occur and that DNA-binding by GR is dispensable. Several mechanistic hypotheses have been advanced. It has been suggested that GR may prevent the binding of cofactors to AP-1, or may compete for limiting amounts of these cofactors. Recent work by de Bosscher and coworkers demonstrates that the levels of p300/CBP in the cell are not limiting and do not contribute to negative crosstalk (De Bosscher et al., 2001).

GR also has been shown to interfere with transcription induced by NF-kB. In part, repression is caused by a GR–mediated increase in transcription of I-kB, the molecule that keeps NF-kB in an inactive cytoplasmic complex (Auphan et al., 1995; Scheinman et al., 1995). However, the modest increase in I-kB protein levels observed upon steroid treatment only partly explains the repression of NF-kB-mediated transcription by GR. In fact, in the absence of I-kB upregulation, GR is still able to repress transcription by NF-kB (Fig. 1Bii) (Heck et al., 1997). Again, a protein-protein interaction between the two transcription factors is important for crosstalk to occur.

The generation of transgenic mice bearing a homozygous mutation in the glucocorticoid receptor leading to a defect in dimerization highlights the importance of these non-genomic transcriptional effects for survival of the organism (Reichardt et al., 1998). Whereas the GR knockout mouse dies shortly after birth, the dimerization defective mutants ($GR^{dim/dim}$) mice are viable and have no overt physical abnormality. Fibroblasts cultures from these mice are unable to generate transcriptional responses from classical GRE-driven promoters such as the mouse mammary tumour virus but are able to repress AP-1-mediated transcription from the collagenase

promoter. Therefore, these mice allow the separation of the direct effects of the glucocorticoid receptor from its non-genomic functions. The viability of this mouse model strongly suggests that the non-genomic modes of action of the receptor play a very important role in development and that direct binding of the receptor to DNA is dispensable for survival of an organism.

Coregulatory Complexes

Eukaryotic chromosomes, forming chromatin, are composed of repeating units of protein-DNA complexes called nucleosomes. Nucleosomes are made up of a histone octamer made up of two units each of histone H2A, H2B, H3 and H4 around which is wrapped 146 base pairs of DNA sequence (Narlikar et al., 2002; Uberbacher and Bunick, 1989). This organization of DNA into nucleosomes creates an energetic barrier against transcription, preventing the association of transcription factors and the RNA polymerase with the DNA substrate. The N-terminal tails of the histone molecules appear to be important in the overall compaction of the DNA because they are responsible for the formation of internucleosomal contacts (Uberbacher and Bunick, 1989). For efficient transcription to occur on chromatin templates, a transcription factor must be able to direct localized changes in the chromatin structure to allow recruitment of the basal transcriptional machinery (Beato and Eisfeld, 1997). Protein complexes containing enzymatic activities that are correlated with gene activation, such as the ability to acetylate histones (histone acetyltransferase activity), create a permissive state of the DNA that can then be remodeled by ATP-dependent protein complexes (Hassig and Schreiber, 1997). Acetylation of lysines in the N-termini of histone tails causes the dissociation of higher order nucleosome structures and increases access for transcription factors and basal transcriptional machinery to DNA. This may be due in part to the charge neutralizing effect of the modification, where ablation of the positive charge of the lysine residues decreases interaction of the histone tail with negatively charged DNA.

Transcription by the liganded GR depends on its interaction with coactivator molecules (Cheung et al., 2000; Glass and Rosenfeld, 2000). These coactivator molecules, normally found in large multi-protein complexes, drive transcription by modifying and remodeling chromatin, and/or by recruiting and /or stabilizing interactions with basal transcriptional machinery (Rachez et al., 1999; Xu and Li, 2003). Coactivators are not specific to GR or to nuclear receptors in general, as these complexes can interact with many different transcription factors (Barlev et al., 2001; Chang et al., 1998; Hung et al., 2001; Sheppard et al., 1999). For a protein to be defined as a coactivator molecule it must be recruited to DNA via a transcription factor, and it must enhance transcription by that factor but not affect basal transcription rates (Robyr et al., 2000).

Corepressor molecules counteract the actions of coactivator molecules in the cell. These corepressors interact with transcription factors and act to deacetylate histone tails and to maintain chromatin in an inaccessible conformation (Xu et al., 1999). Transcription in the cell is thus a result of coordinated inputs from coactivators and corepressor molecules. Cell signaling events, such as cortisol secretion, can cause a shift in the balance of coactivators and corepressors found at a specific promoter leading to changes in transcription rates. Regulation by phosphorylation, acetylation, methylation and even targeted proteolysis have been described as methods of regulating transcriptional responses (Grozinger and Schreiber, 2000; Kouzarides, 2000; Kuo and Allis, 1998; Spencer and Davie, 1999). To further add complexity, many coregulatory molecules display tissue-specific distribution and dissimilar protein levels within different cell types of a tissue (Xu and Li, 2003). This complexity may explain how a gene is regulated differently in various cell types.

Nuclear receptors such as the retinoic acid receptor and the thyroid receptor are always localized to the nucleus of a cell (Lin et al., 1991; Strait et al., 1991). Thus, even under conditions when the appropriate hormone is not present, these receptors are able to interact with their cognate DNA elements and theoretically could influence gene expression. To prevent transcription in the absence of hormone, these receptors are complexed with large multi-protein

corepressor complexes that contain deacetylase activity (Xu et al., 1999). This activity prevents acetylation of histone tails and premature remodeling of the chromatin structure. Upon hormone binding, a conformational change occurs in the last helix of the ligand binding domain (the AF-2 region) creating a surface that no longer supports interactions with corepressor molecules (Perissi et al., 1999; Renaud et al., 1995; Wagner et al., 1995). A coactivator-containing complex replaces the corepressor complex and chromatin remodeling can occur (Moras and Gronemeyer, 1998).

GR, in the absence of hormone binding is cytoplasmic (Picard and Yamamoto, 1987; Sackey et al., 1996). By virtue of this localization in the cell, it is unable to interact with DNA in the absence of hormone binding. Only upon ligand binding does the receptor translocate to the nucleus where it can interact with coactivator complexes via its AF-2 domain.

Histone acetyltransferases (HAT)

Histone acetyltransferases can be subdivided into two families. One subclass (Type B) is cytoplasmically localized and acetylates newly translated histones. The modification allows for transport of the histones into the nucleus (Ruiz-Carrillo et al., 1975). The second subclass (Type A), composed of the histone acetyltransferases that influence transcription, is located in the nucleus and acetylates nucleosomal histones.

Type A histone acetyltransferases have a highly conserved structure, which includes the catalytic domain and a bromodomain. The bromodomain has been implicated in binding acetylated lysine residues and may serve to target the acetylase to chromatin (Dhalluin et al., 1999; Owen et al., 2000).

p160 coactivator family

The p160 coactivator family is a family of evolutionarily related proteins. Currently there exist five members of the family, many with various isoforms of unknown function (Cavailles et al., 1994; Halachmi et al., 1994). Due to their characterization by several different laboratories, each p160 member has multiple common names as well as the universal classification of NCoA for nuclear receptor coactivator. The NCoA family includes the prototypical member NCoA1/SRC-1 (steroid receptor coactivator 1), NCoA2/GRIP1, NCoA3/p/CIP, NCoA4/ARA70 and NCoA6/Asc2 (Anzick et al., 1997; Onate et al., 1995; Torchia et al., 1997; Voegel et al., 1996). The p160 family is characterized by its ability to interact with a large number of nuclear receptors through the AF-2 region. The interaction with the ligand binding domain of nuclear receptors is ligand dependent. The p160 recognizes a conserved interaction surface formed by the hormone-dependent conformational change of the last helix. The "NR box" of the coactivator, an LxxLL motif, is used to interact with the nuclear receptor by recognition of a hydrophobic cleft in the AF-2 and is stabilized in position by a "charge clamp" of conserved glutamate and lysine residues within the ligand binding domain (Nolte et al., 1998). Many p160 coactivators have multiple LxxLL motifs and use them differently to contact the various nuclear receptors (Ding et al., 1998). For example, GR has a preference for NR box II of NCoA2/GRIP1, and this preference has recently been shown to be due to a unique second charge clamp present in the ligand binding domain of GR (Bledsoe et al., 2002).

NCoA1/SRC-1 was the first member of the p160 family cloned (Onate et al., 1995). This protein contains interaction motifs for association with nuclear receptors and can interact with the transcriptional coactivator p300/CBP via a separate domain (Yao et al., 1996). Despite a demonstration of weak histone acetyltransferase activity in this protein and for NCoA3/p/CIP, it appears to function more as a scaffold for the building of coactivating complexes on nuclear

receptor ligand binding domains (Spencer et al., 1997). The genetic ablation of NCoA1/SRC-1 in mice produces no overt phenotype suggesting that the functions of SRC-1 are redundant *in vivo* (Xu et al., 1998a). However, fibroblasts taken from these mice display steroid and thyroid hormone resistance suggesting that SRC-1 is an important regulator of lipophilic hormone action. Furthermore, SRC-1 has been shown to mediate transcription by non-receptor transcription factors such as AP-1, NF-kB and p53 (Lee et al., 1999; Lee et al., 1998; Na et al., 1998). This role for SRC-1 may suggest a mechanism for transcriptional crosstalk between the glucocorticoid receptor and AP-1 and NF-kB, where liganded GR could compete with the DNA-bound transcription factors for SRC-1, thereby limiting the transcriptional response (Sheppard et al., 1998).

p300/CBP

p300 was originally purified as the cellular binding protein for the adenoviral protein E1A (Eckner et al., 1994). The closely related protein CBP was originally purified as a factor associated with the cAMP response element binding protein CREB (Chrivia et al., 1993). p300/CBP share 63% amino acid identity over their length, but have much greater similarity in the domains they share such as the CREB binding site, the E1A binding site and the bromodomain (Arany et al., 1994). After genetic ablation of p300, mice die during gestation due to defects in heart development and cell proliferation, suggesting that CBP is not able to rescue the role of p300 in the embryo (Yao et al., 1998). Similar results were obtained in mice deficient for CBP, and in fact heterozygosity of either factor was associated with high embryonic lethality. Gene dosage of these transcriptional coactivators is essential for development and survival.

Both p300 and CBP possess powerful histone acetyltransferase activities that are able to acetylate histone targets such as histone H3 and H4 as well as many non-histone targets (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). These non-histone targets include the

basal transcription factor TFIIEβ, p53, GATA-1, NCoA3/p/CIP and HMG-1(Y) (Boyes et al., 1998; Chen et al., 1999; Gu and Roeder, 1997; Imhof et al., 1997; Munshi et al., 1998). Both p300 and CBP can potentiate transcription by nuclear receptors, and in the case of the estrogen receptor, the enhancing effect on transcription occurs only on chromatin templates (Kraus and Kadonaga, 1998). These results indicate that the major role of p300/CBP may be to open chromatin structure by acetylation of histone tails. The large multi-domain structure of these coactivators also allows them to act as bridges between DNA-bound transcription factors and the basal transcriptional machinery, as well as providing a link between transcription factors and other coactivator molecules (Chan and La Thangue, 2001).

GNAT

The GCN5-related N-acetyltransferases (GNAT) family of acetyltransferases includes GCN5, the p300/CBP associated factor PCAF and the more distantly related Hat1, Elp3, and Hpa3 (Sterner and Berger, 2000). The family is grouped into sub-families based on sequence similarity over four conserved domains, and all are related to the yeast protein GCN5. Mammalian GCN5 and PCAF are extremely similar in sequence, though mammalian PCAF was thought to encode an N-terminal extension of approximately 350 amino acids. Recently, a GCN5 was cloned from both mice and humans that is more similar in length to PCAF. This GCN5 and PCAF share approximately 70% amino acid identity and upwards of 80% similarity (Xu et al., 1998b). The shorter form of GCN5 lacking the N-terminal extension found in both humans and mice appears to be the result of inefficient splicing of the mRNA transcript. The N-terminus of the protein appears to increase the specificity of GCN5 to include nucleosomal histones, a substrate previously thought to only be efficiently acetylated by PCAF (Xu et al., 1998b; Yang et al., 1996b). Both proteins are able to interact with p300/CBP and form part of large coactivating complexes associated with nuclear receptors (Xu et al., 1998b; Yang et al., 1996b). Interestingly,

despite an ability to regulate transcription via their HAT activity and a demonstrated ability to acetylate histones H3 and H4, PCAF has been extensively implicated in the acetylation of many non-histone targets such as p53 and MyoD (Liu et al., 1999; Sartorelli et al., 1999; Wang et al., 1997; Yang et al., 1996b). Non-chromatin acetylation by GCN5 has only been shown for c-myb (Tomita et al., 2000).

Both PCAF and GCN5 function as part of large distinct multiprotein complexes that share many of the same subunits related to the yeast SAGA complex. These include members of the basal transcriptional machinery like the TATA binding protein associated factors (Bhaumik and Green, 2002; Brand et al., 1999; Forsberg et al., 1997; Imhof et al., 1997). Although PCAF has been shown to interact with the AF-1 of certain nuclear receptors, it is also recruited to the AF-2 region in the presence or absence of an interaction with p300/CBP and p160 coactivators (Blanco et al., 1998; Korzus et al., 1998). GCN5 has not yet been shown to interact with nuclear receptors in this fashion but is a component of a coactivating complex required for estrogen receptor transcriptional activity (Yanagisawa et al., 2002).

GCN5 and PCAF are widely expressed in mice, but their comparative levels vary greatly in different tissues (Xu et al., 1998b). Genetic ablation of PCAF does not result in any overt phenotype whereas the loss of GCN5 in mice is embryonic lethal, indicating that PCAF is not able to functionally replace GCN5 *in vivo* (Yamauchi et al., 2000). The GCN5 null animals show severe growth retardation and fail to form dorsal mesodermic lineages due to an increase in apoptosis. Deletion of both GCN5 and PCAF leads to even more severe abnormalities than the GCN5 null phenotype alone, and indicates that PCAF can compensate for loss of GCN5 in overlapping functions.

Histone Deacetylases (HDAC)

The deacetylation of histones is thought to cause the repression of transcription by stabilizing chromatin to inhibit the unwinding of the promoter region, and the association of activating factors (Narlikar et al., 2002; Strahl and Allis, 2000). Proteins possessing deacetylase activity can be divided into two families. The NAD^+-dependent deacetylases, known as sirtuins, are more closely related to the yeast protein Sir2 (Imai et al., 2000). The family comprises 7 members that are localized to the cytoplasm (Afshar and Murnane, 1999) where their cellular role is yet to be defined. Recently, a member of this family, SIRT1, was shown to interact with and deacetylate p53, repressing its ability to activate transcription in response to DNA damage (Vaziri et al., 2001).

The second family of histone deacetylases has eleven members that fall into one of two classes. Class I HDACs include HDAC1, 2, 3 and 8 and are most closely related to the yeast deacetylase RPD3, originally identified as a transcriptional repressor in that organism. These HDACs are mainly localized to the nucleus and are widely expressed in mammalian tissues.

Class II HDACs share more similarity with the yeast deacetylase HDA1, and appear to have more tissue specific expression patterns. HDAC4, 5, 6, 7, 9 and 10, forming class II, are larger proteins than class I deacetylases by virtue of a large N-terminal domain. They are regulated by nucleocytoplasmic shuttling and controlled by the phosphorylation status of the protein. For example, in undifferentiated muscle cells, HDAC4 is phosphorylated by Ca^{2+}/calmodulin-dependent kinase which causes its export to the cytoplasm by the export receptor CRM1. In the cytoplasm, phosphorylated HDAC4 is bound by 14-3-3 proteins thereby preventing its re-entry into the nucleus. Differentiation signals cause a decrease in HDAC4 phosphorylation triggering the release of HDAC4 from 14-3-3 and nuclear localization (Miska et al., 1999)

HDAC11, the most recently identified HDAC, does not bear significant homology to either class of HDAC, and may represent a new class of HDAC protein (Gao et al., 2002). Its precise role in controlling transcription remains unknown.

HDAC Complexes

Hassig and coworkers used a trapoxin affinity matrix to precipitate HDAC1, 2 and 3 from HeLa cell extract (Hassig et al., 1998). The precipitate was able to deacetylate all four core histones, and recombinant HDAC1 could efficiently deacetylate both free and nucleosomal substrates *in vitro*. It was observed that the HDAC1 and 2 coprecipitated while HDAC3 formed part of a separate complex.

In fact, mammalian HDAC1 and 2 form part of large multiprotein complexes in the cell. These complexes, called the NuRD (nucleosome remodeling and deacetylase complex), the Sin3 (named after its core component mSin3A) and the CoREST complexes share the deacetylase components HDAC1 and HDAC2 but differ in the other molecular components (Ayer, 1999; You et al., 2001). The NuRD complex has seven subunits: Mi-2, MTA2, RbAp46, RbAp48, methyl CpG binding domain protein 3 (MBP3) and the deacetylases (Zhang et al., 1999). The RbAp46 and RbAp48 components were originally identified through binding to the retinoblastoma protein (Qian et al., 1993). *In vitro*, RbAp46 can bind directly to helix 1 of histone 4 and may act to recognize chromatin targets (Verreault et al., 1998). In addition to the deacetylase activity of this complex, the NuRD complex can also actively remodel chromatin via the Swi2/Snf2 helicase activity of the Mi-2 component (Zhang et al., 1999). This remodeling activity may be necessary for histone deacetylases to reach their histone targets efficiently.

The Sin3 complex also contains the core proteins HDAC1 and 2 and RbAp46 and RbAp48. The complex also contains the unique members mSin3A and its associated proteins SAP10, and SAP30 (Zhang et al., 1998b). SAP30 interacts directly with corepressor protein

NCoR and may anchor the corepressive complex to unliganded nuclear receptors. Recently three new components of the Sin3 complex, SAP180, SAP130 and SAP45 were identified (Fleischer et al., 2003). All three components could repress transcription when tethered to DNA, and form functional components of the complete complex. The Sin3 complex is unable to deacetylate nucleosomal histones *in vitro*, suggesting the need for accessory factors for its activity. In fact, the Sin3 complex is targeted to promoters via DNA-binding factors such as unliganded nuclear receptors, the Mad/Max heterodimer and p53 (Ayer et al., 1995; Murphy et al., 1999; Soderstrom et al., 1997)

The CoREST complex differs in its core composition in that it lacks both RbAp48 and 46 and includes a corepressor protein CoREST (You et al., 2001). The role of the CoREST deacetylase complex in the cell is unknown, though recently it has been implicated in the repression of neuronal gene expression (Battaglioli et al., 2002).

HDAC1

HDAC1 is a nuclear protein by virtue of a lysine rich nuclear localization signal found in the C-terminal tail of the protein and a lack of a corresponding export signal (Johnstone, 2002; Taplick et al., 2001). The catalytic core of a bacterial class I HDAC from *Aquifex aerolicus* was crystallized bound to the HDAC inhibitor trichostatin A (Finnin et al., 1999). This protein shares 35.2% identity with human HDAC1. The catalytic core of the protein spans 390 amino acids and represents the majority of the protein. The active site forms a wide-bottomed tubular pocket that includes a zinc-binding site. The inhibitor contacts several conserved residues within the active site, blocking the entry of an acetylated substrate. The removal of the acetyl group occurs through a charge-relay system consisting of two aspartate residues separated by six amino acids found 30 residues downstream of two adjacent histidine residues. A tyrosine residue located 120 amino acids downstream of the aspartates is also critical for deacetylation.

HDAC1 is induced by interleukin-2 in murine T cells allowing cells to proliferate in response to this cytokine. In fact, HDAC1 expression is low in quiescent cells and increases as cells progress through the G1/S boundary (Bartl et al., 1997). Genetic ablation of HDAC1 in the mouse resulted in embryonic lethality due to severe proliferation defects (Lagger et al., 2002). Taken together, these results indicate an important role for HDAC1 in cell-cycle progression.

HDAC1 is also the target of phosphorylation by casein kinase 2 at serine residues 421 and 423 of the human protein. Mutation of the phosphorylated serines to alanine residues decreased the enzymatic activity of the protein and disrupted complex formation with RbAp48, mSin3A, MTA2 and CoREST (Pflum et al., 2001).

HDAC1 has also been described to act independently of corepressor complexes, as a free deacetylase. HDAC1 can interact with and repress transcription by YY1, Sp1, and by MyoD (Yao et al., 2001). Interaction between HDAC1 and Sp1 prevents Sp1 interaction with E2F1 thereby preventing the expression of S-phase genes positively regulated by E2F1 (Doetzlhofer et al., 1999). Transcriptional activation by MyoD is prevented in undifferentiated muscle cells through its association with HDAC1 (Mal et al., 2001).

Nuclear Hormone Receptors and Histone Deacetylases

The silencing of gene activation by unliganded nuclear receptors such as the thyroid hormone receptor and the retinoic acid receptor led to a model in which the transcriptionally inactive state was achieved by the recruitment of corepressor proteins possessing histone deacetylase activity, which prevent the opening of chromatin to basal transcriptional machinery. Ligand binding and the associated conformation change would then cause the release of the corepressor proteins and allow the interaction of coactivating complexes. In fact, crystal structures of the unliganded PPARα receptor revealed that corepressor complexes interact with the unliganded nuclear receptor through a conserved motif of LxxI/HIxxxI/L (Xu et al., 2002).

This consensus motif is an extended version of the interaction domain used by coactivator molecules. Binding of ligand, and formation of the charge clamp does not support interaction of corepressor molecules and may provide a mechanistic explanation of how nuclear receptors switch from being repressive to activating on chromatin templates (Xu et al., 2002).

Two corepressor proteins, identified separately by their interactions with unliganded thyroid hormone receptor and retinoic acid receptor, serve as anchor points for large multiprotein complexes to assemble on unliganded receptors. These proteins, NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic and thyroid hormone receptor) share significant sequence similarity in both their N and C-termini (Chen and Evans, 1995; Horlein et al., 1995; Kurokawa et al., 1995). Both proteins are specifically recruited to unliganded TR and RAR but are not recruited to members of the steroid hormone subfamily or the vitamin D receptor. Both proteins encode repressor domains and have domains used to interact with nuclear receptors, and these are used differentially depending on the recruiting receptor (Wong and Privalsky, 1998b). The repressive domains of NCoR and SMRT can associate with histone deacetylases (HDACs) to maintain histone hypoacetylation at chromatin targets.

It is important to note that steroid receptors have no DNA binding activity in the absence of hormone, and do not interact appreciably with either corepressor protein. There is little evidence to suggest that GR or other steroid receptors interact with corepressor molecules in the presence of steroid. However, the association of corepressor molecules NCoR and SMRT with the partial agonist tamoxifen-bound estrogen receptor has been observed *in vitro* (Smith et al., 1997; Zhang et al., 1998a). In the presence of antagonists, such as the anti-estrogens or anti-progestins, the estrogen and progesterone receptor has been shown to interact with SMRT (Jackson et al., 1997; Smith et al., 1997). The antagonist bound glucocorticoid receptor has been shown to interact with NCoR, but the *in vivo* significance of this interaction is unknown (Schulz et al., 2002).

GR has been shown to recruit HDAC2 to a NF-kB (p65) complex in the absence of NCoR or SMRT and thereby repress interleukin-1β induced gene expression. The interaction between GR and HDAC2 is agonist dependent and only observed when low concentrations of dexamethasone are used (Ito et al., 2000). The antagonist, mifepristone (RU486) can also inhibit p65-associated histone acetyltransferase activity (Ito et al., 2001). The recruitment of the deacetylase by activated GR could provide a mechanism for the crosstalk observed between GR and NF-kB.

Regulation of transcription factors by acetylation and deacetylation

Modification by acetylation is not reserved to histones and components of chromatin. Many transcription factors have recently been shown to be targets of acetylation by p300/CBP, PCAF, or GCN5 or combinations thereof and this covalent modification regulates their transcriptional activation potential.

For many transcription factors acetylation has been shown to influence the DNA-binding activity. Acetylation of p53 by PCAF and p300/CBP results in an increase in DNA-binding by the transcription factor and a positive effect on p53-mediated transcription (Gu and Roeder, 1997; Liu et al., 1999). Similar regulation is seen for GATA-1 during differentiation of the hematopoietic system. GATA-1 interacts with p300/CBP *in vivo*, and is acetylated by p300 *in vitro* increasing its DNA-binding capacity (Boyes et al., 1998).

In addition to enhanced DNA binding, the acetylation of cell cycle regulator E2F1 by PCAF increases the protein's half-life (Martinez-Balbas et al., 2000). Although the exact mechanism is unknown; it is possible that PCAF and a member of the ubiquitin-proteasome system compete for modification of the same lysine residues. Acetylation would then prevent ubiquitylation, and reduce the turnover of E2F1 protein. This type of regulation by acetylation has been observed for the inhibitory transcription factor Smad7. Smad7 associates with the

ubiquitin ligase Smurf1, and upon transforming growth factor β (TGFβ) signaling, the complex translocates from the nucleus of the cell to the plasma membrane where Smurf1 ubiquitylates the TGFβ receptor, attenuating signaling. Smad7 is also effectively ubiquitylated by Smurf1 and is targeted for degradation by the 26 S proteasome. Acetylation of Smad7 by p300 on lysines that are also targeted for ubiquitylation reduces protein turnover (Gronroos et al., 2002). TGFβ signaling therefore reduces the acetylation of Smad7, making the lysine residues available for modification by Smurf1 having the overall effect of reducing the repressive actions of Smad7 on transcription.

The acetylation of transcription factors can also influence transcription by modulating the interaction of the factor with coregulatory molecules. In the case of p53, its acetylation facilitates the recruitment of the coactivator proteins CBP and TRRAP, leading to induction of p21 transcription (Barlev et al., 2001). In the absence of acetylation, coactivator interactions are reduced and transcription of p21 by p53 is limited. Even the coactivators themselves are subject to regulation by acetylation. For example, acetylation of NCoA3/p/CIP by p300/CBP on lysine residues adjacent to the nuclear receptor interaction domain disrupts the interaction with the estrogen receptor and leads to the attenuation of the transcriptional response by causing the dissociation of the HAT complex (Chen et al., 1999).

Given that histone acetyltransferases can acetylate non-histone targets, it is proposed that HDACs can function as factor deacetylases to counterbalance the effects of PCAF, GCN5 and p300/CBP (Chen et al., 2001; Sterner and Berger, 2000). The association of transcription factors with deacetylase activity could influence not only chromatin structure but also directly impact transcription factor activity. For example, transcription factor MyoD is acetylated by PCAF upon induction of the muscle differentiation program and this modification increases MyoD's transcriptional activation of muscle specific genes. In the absence of differentiation signals, MyoD is found directly associated with HDAC1 in an unacetylated state. Though *in vivo*

demonstration of HDAC1's factor deacetylase activity is lacking, HDAC1 could deacetylate MyoD *in vitro* (Mal et al., 2001).

The regulation of transcription factor activity through acetylation of the factor itself or coregulatory molecules increases the complexity of transcriptional regulation beyond the modification of histones. Whereas the acetylation of histones is associated with the opening of chromatin, acetylation of transcription factors, though mostly associated with increased transcriptional activity, can, as in the case of the acetylation of NCoA3/p/CIP results in the attenuation of a transcriptional response.

Regulation of Transcription by Ubiquitylation

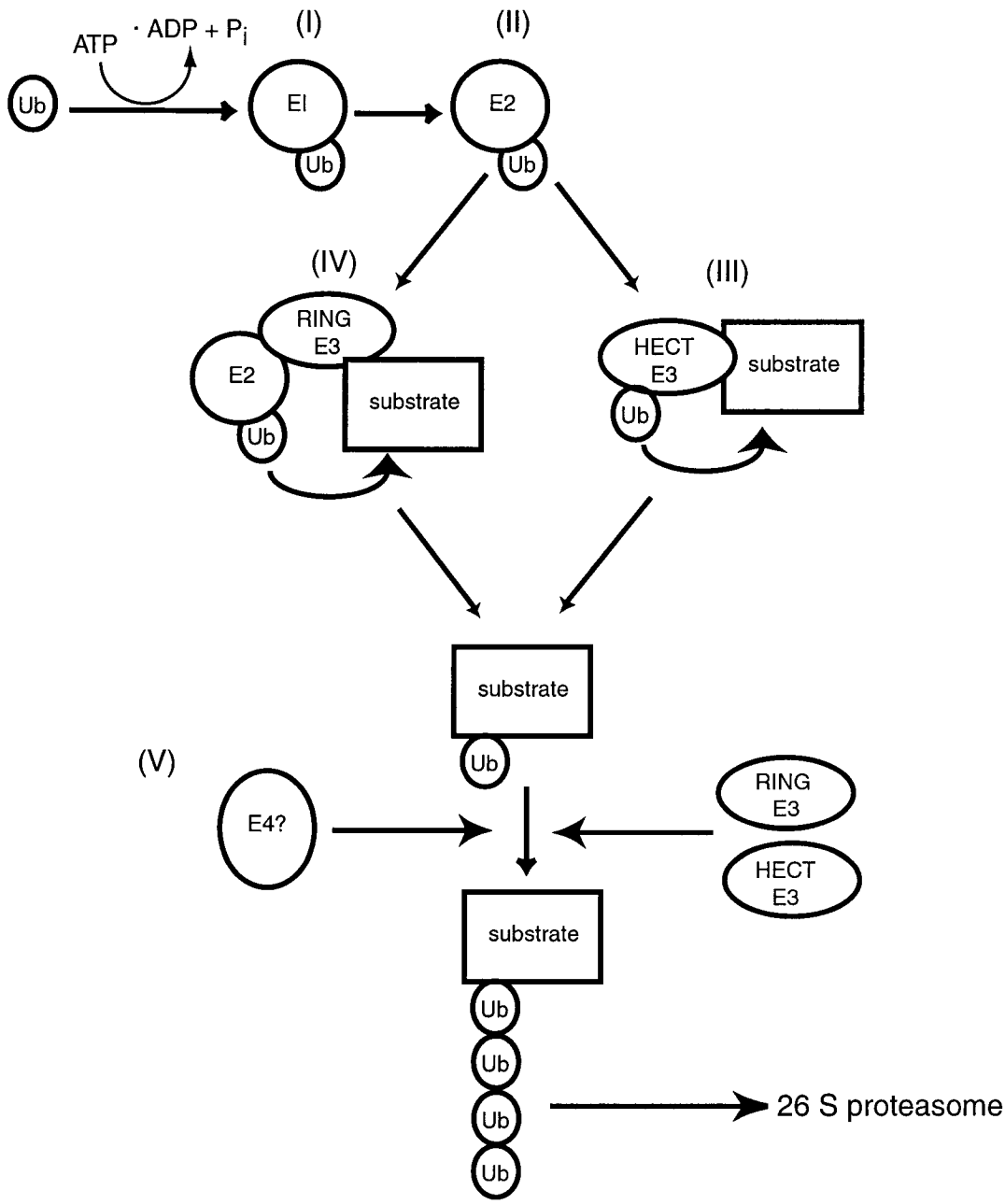
Ubiquitin Conjugation

Ubiquitin is a highly conserved molecule of 76 amino acids found in all mammalian cells (Pickart, 2001). It is covalently linked to lysine residues of proteins within the cell. The addition of long ubiquitin chains is associated with targeting to the 26 S proteasome for degradation. Monoubiquitylation events have also been described for proteins such as p53 and histones, though the impact of such modifications is still unknown but may play a role in membrane transport and perhaps transcriptional regulation (Chau et al., 1989; Lai et al., 2001; Spencer and Davie, 1999; Thrower et al., 2000).

To target a protein for degradation by the proteasome, it must first be conjugated to a chain of at least four ubiquitin molecules via a lysine residue (Chau et al., 1989; Thrower et al., 2000). Conjugation of a target protein requires the activity of a cascade of enzymes (Fig. 2). First, an E1 ubiquitin activating enzyme activates the ubiquitin monomer by ATP hydrolysis and links the moiety covalently to a cysteine residue within the catalytic site of the enzyme (Ciechanover et al., 1982). The activated ubiquitin moiety is then transferred to a cysteine residue of an E2 ubiquitin conjugating enzyme (Hershko et al., 1983). There exist approximately

Figure 2. Targeting of a protein substate for degradation by the ubiquitin-proteasome system.

Through the hydrolysis of ATP, a ubiquitin moiety (Ub) is transferred to the E1 ubiquitin activating enzyme active site (I). The activated ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme (II). Transfer of the ubiquitin moiety to a HECT family E3 ubiquitin ligase active site results in the transfer of the Ub to the recognized substrate (III) . Alternatively, the E2 ubiquitin conjugating enzyme can interact with an RING E3 ubiquitin ligase-substrate complex. The ubiquitin moiety is then transferred directly from the E2 enzyme to the substrate protein (IV). Elongation of the ubiquitin chain, necessary for recognition of the substrate by the 26 S proteasome, is accomplished by an E3 ubiquitin ligase or via a novel E4 elongating enzyme (V).


ATP
· ADP + Pi
(I)
(II)
Ub
E1
Ub
E2
Ub
(IV)
(III)
RING
E3
E2
Ub
substrate
HECT
E3
Ub
substrate
substrate
Ub
(V)
E4?
RING
E3
HECT
E3
substrate
Ub
Ub
Ub
Ub
26 S proteasome

20 known E2 enzymes within the cell (Pickart, 2001). To complete conjugation of ubiquitin onto a cellular target protein an E3 ubiquitin ligase is required. The role of the E3 ubiquitin ligase is to recognize the appropriate target and thus there exist many characterized E3 enzymes within the cell (Hershko et al., 1983). These enzymes provide specificity to the ubiquitin conjugating system. E3 ubiquitin ligases fall into two classes, the RING E3 and the HECT E3, based on the presence of conserved domains. Although both classes catalyze the transfer of ubiquitin to lysines on target proteins, they do so in very different ways. The RING finger E3 ubiquitin ligases, for example Mdm2, are characterized by the presence of a RING (really interesting new gene) finger domain and can function alone or as part of a complex using cullin family members as components of the active complex (Pickart, 2001). These E3 ubiquitin ligases recognize their specific target and transfer the ubiquitin moiety from the E2 enzyme directly to the target lysine (Lorick et al., 1999). On the other hand, HECT E3 enzymes are characterized by homology to ubiquitin ligase E6-AP (Huibregtse et al., 1995; Pickart, 2001). These proteins transfer the ubiquitin moiety from the E2 enzyme to an internal catalytic cysteine before transferring the molecule to the target protein lysine (Huibregtse et al., 1995). The formation of multi-ubiquitin chains necessary for targeting to the proteasome occurs by an unknown mechanism. It has been demonstrated that the higher order ubiquitin chains can be produced by the RING finger E3 ubiquitin ligases, though the strict substrate specificity of other E3 enzymes may prevent them from recognizing the highly conserved lysine structure of ubiquitin itself (Pickart, 2001; Xie and Varshavsky, 1999). Recently, an "E4" enzyme has been characterized (Hatakeyama and Nakayama, 2003). This enzyme is believed to be responsible for adding further ubiquitin moieties to target proteins monoubiquitylated by the appropriate E3 ubiquitin ligase (Fig.2).

Ubiquitylation and Transcription

Although the role of the ubiquitin-proteasome system within the cell is well characterized, its role in an intimate link with the process of gene activation is new. Recently, Muratani and Tansey proposed that the ubiquitin system acts to control transcription by recruitment of the 26 S proteasome to the promoters of active genes (Muratani and Tansey, 2003). Since many acidic transcriptional activation domains have been shown to also act as sequences that drive the destruction of the activator (degrons), they propose that the recruitment of members of the ubiquitin pathway to the promoters of active genes would lead to the ubiquitylation of not only the transcription factor, but also histone targets (such as histone H2B), RNA polymerase II and possibly recruited coactivator molecules (Brower et al., 2002; Kim and Maniatis, 1996; Lo and Massague, 1999; Muratani and Tansey, 2003; Salghetti et al., 2001; Salghetti et al., 2000). Indeed, the potency of an activation domain has been inversely linked to protein stability but only when linked to an intact DNA-binding domain (Molinari et al., 1999). This mechanism would ensure the rapid shutdown of transcription by making the activation of transcription self-limiting.

Many transcription factors are targets of activation-induced degradation. The transcription factor p53 is rapidly degraded following nuclear localization by the RING finger E3 ubiquitin ligase Mdm2, and this degradation inhibits its transcriptional activity (Haupt et al., 1997; Kubbutat et al., 1997). In contrast to this negative regulation, the activity of NF-kB is positively regulated by the ubiquitin-proteasome system. NF-kB is kept sequestered in the cytoplasm of the cell by an inhibitory binding partner I-kB (Baeuerle and Baltimore, 1988). Upon proinflammatory signaling, I-kB is ubiquitylated and degraded by the 26 S proteasome (Palombella et al., 1994). NF-kB is then free to enter the nucleus of the cell and influence transcription of its target genes. In fact, the inactive preprotein forms of NF-kB may require proteolytic processing by the ubiquitin-proteasome system to become the active isoform.

Ubiquitylation of the inactive p105 and p100 isoforms directs proteolytic cleavage of the precursors to the active p52 and p50 forms (Palombella et al., 1994).

Preadipocyte Differentiation

In times of caloric restriction, an organism relies on stores of fat to provide the energy necessary for survival. This energy, stored during times of caloric excess, is found in the form of lipid in specialized adipocyte cells. Adipocytes are capable of storing large amounts of lipid, and when caloric intake far exceeds the energy requirement of the organism, can increase in size to accommodate the excess lipid. If the caloric excess is prolonged, new adipocytes are formed through the differentiation of fibroblastic precursor cells.

Models of preadipocyte differentiation

The best-studied models for preadipocyte differentiation are derived from day 17 mouse embryos. The NIH 3T3 cell line, a pluripotent fibroblastic cell line, can be differentiated into the adipocyte, myoblast or chondrocyte lineage depending on the hormonal stimulus provided (Yeh et al., 1995). 3T3 L1 and 3T3 F442A cells are both committed to the adipocyte lineage, but 3T3 L1 cells are the most used model because they differentiate in a synchronous manner in response to insulin, cAMP signaling and glucocorticoids whereas 3T3 F442A cells are further along the differentiation pathway and only require insulin to differentiate (Moustaid et al., 1990; Rubin et al., 1978). 3T3 L1 cells are then ideal to study the early transcriptional events of the differentiation process. This cell line also accurately represents the formation of fat pads *in vivo*, as injection of these preadipose cells into immunocompromised mice leads to the development of white fat depots that are histologically identical to the natural fat (Green and Meuth, 1974). A further validation of the 3T3 L1 model of preadipocyte differentiation is provided by the use of

primary cultures from various mammalian species. These primary cultures are morphologically fibroblastic as are the 3T3 L1 cells, and differentiate in culture in response to insulin and for cultures derived from mice, pigs and humans, glucocorticoids (Gregoire et al., 1998). Glucocorticoids are present in the hormonal inducing cocktail for the first 48 hours of the differentiation program after which they are removed (Rubin et al., 1978). The continuous presence of glucocorticoids in the differentiation cocktail beyond 48 hours does not further enhance differentiation. Although glucocorticoids are not strictly required for preadipocyte differentiation, they provide a strong enhancing effect, with cultures producing up to 10-fold more adipocytes (Green and Kehinde, 1975).

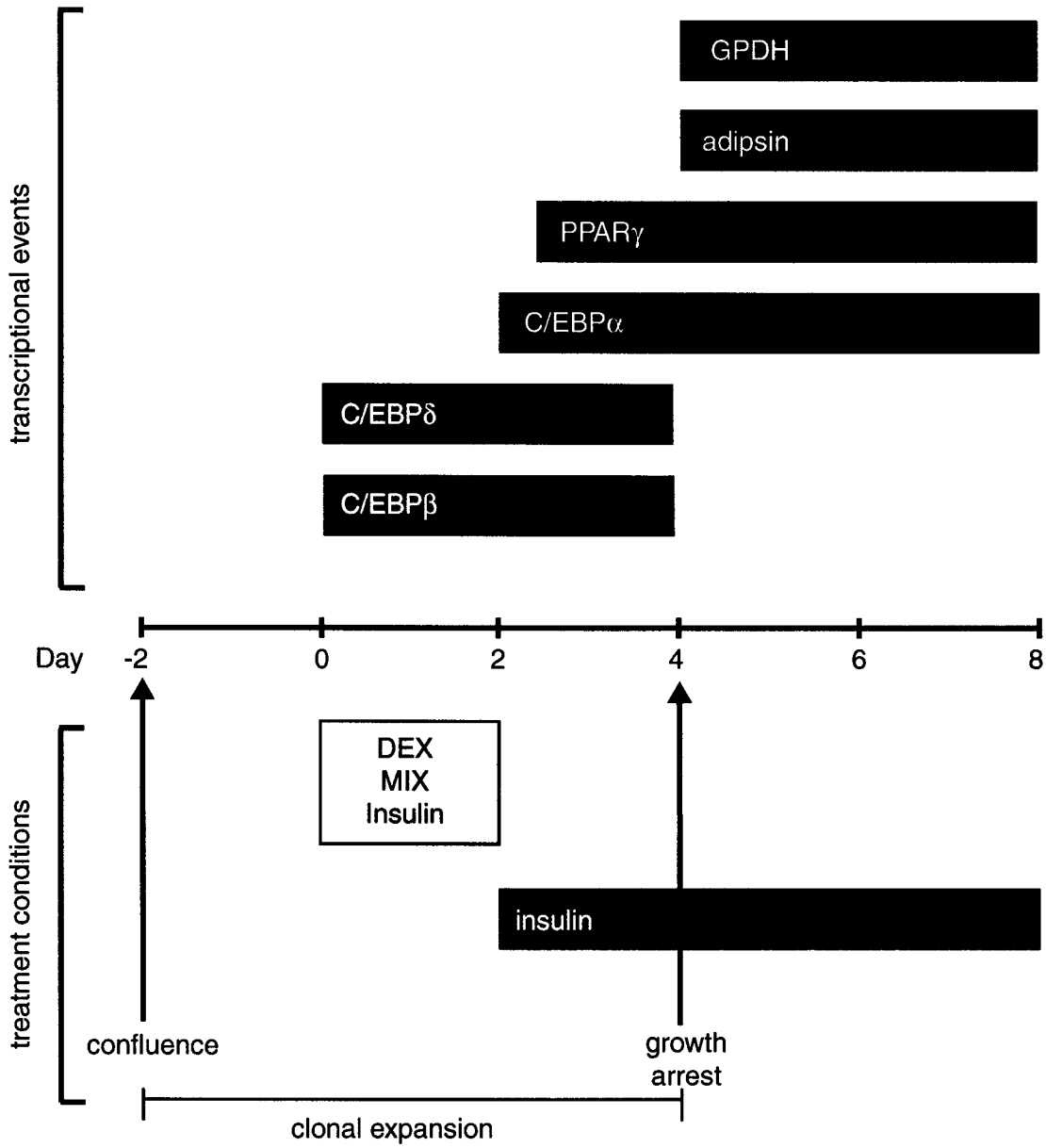
The differentiation of preadipocytes into adipocytes *in vitro* occurs following the activation of a transcription factor cascade by hormonal stimulation (Fig. 3). Initially, members of the CCAAT/enhancer binding proteins (C/EBPs) drive differentiation-specific transcription. Their actions are complemented by the actions of the peroxisome proliferator activated receptor γ (PPARγ).

CCAAT/enhancer Binding Proteins

C/EBPs are a family of bzip transcription factors that arose from gene duplication events (Lekstrom-Himes and Xanthopoulos, 1998). There are six family members, C/EBPα, C/EBPβ/LAP/NF-IL6, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ/CHOP. Their carboxy-terminal basic DNA-binding domains and leucine zipper motif, collectively known as the bzip domain, characterize the family. The family members vary in their abilities to activate transcription, and due to their important roles in differentiation processes, their activity is tightly regulated. C/EBPs can homo-and heterodimerize, a prerequisite for DNA-binding, increasing the variation of their transcriptional responses (Landschulz et al., 1989). Although a consensus DNA motif has been described for these factors, they are able to bind highly variable sequences. C/EBPα and β have

Figure 3. Transcriptional cascade of preadipocyte differentiation of 3T3 L1 cells.

Differentiation of 3T3 L1 preadipocytes is a 10 day process that is initiated when cells reach confluence. This day is known as day –2. Two days post-confluence (day 0) cells are treated with a hormonal cocktail containing dex, MIX and insulin for 48 hrs. This treatment leads to the rapid upregulation of C/EBPβ and δ proteins, whose levels peak at 24 hrs post-treatment and slowly decrease thereafter, and with the clonal expansion. C/EBPβ and δ in turn activate the transcription of the C/EBPα gene, whose expression slowly increases to a maximum at approximately day 4. Expression of C/EBPα remains high throughout terminal differentiation due to autoregulation of the gene. The appearance of C/EBPα corresponds with growth arrest of the cultures. C/EBPα expression can then activate the transcription of PPARγ, which can auto and cross-regulate its own gene and the C/EBPα gene. The expression of both C/EBPα and PPARγ leads to the expression of adipocyte markers such as adipsin and GPDH.


transcriptional events
GPDH
adipsin
PPARγ
C/EBPα
C/EBPδ
C/EBPβ
Day
-2
0
2
4
6
8
treatment conditions
DEX
MIX
Insulin
insulin
confluence
growth
arrest
clonal expansion

been shown to be located to centromeric satellite DNA due to repetitive stretches of C/EBP consensus motifs in this DNA (Tang and Lane, 1999).

C/EBPβ is expressed in liver, intestine, lung and adipose tissue and its expression can be induced by lipopolysaccharide, interleukin-6, and interleukin-1 (Akira et al., 1990; Poli et al., 1990). A predominantly nuclear protein, this localization can be promoted by tumour necrosis factor α (Yin et al., 1996). The activity of C/EBPβ is regulated by multiple translational starts and post-translational modifications. Differential initiation of translation results in 3 C/EBPβ proteins with identical carboxy termini, and truncated amino termini. The full-length isoform, also known as Liver Activating Protein (LAP) is an activator of transcription, as it contains all three of the activation domains. The second isoform lacks only the first 21 amino acids but contains all of the activation domains. Although the truncated region may be important in stabilizing interactions with cofactors, this C/EBPβ isoform acts as a transcriptional activator (Kowenz-Leutz and Leutz, 1999; Mink et al., 1997). The shortest of these isoforms produces protein that lacks any activation domains and therefore acts as a dominant negative protein known as Liver Inhibitory Protein (LIP) (Descombes et al., 1990; Ossipow et al., 1993). Dimerization of this isoform with full length C/EBPβ results in a dampening of the transcriptional activation by the transcription factor. Overexpression of this isoform can disrupt differentiation of 3T3 L1 preadipocytes.

In addition to translational control of C/EBPβ activity, C/EBPβ is also post-translationally modified to further modulate its activity. Though predominantly a nuclear protein, phosphorylation of a serine residue within the nuclear localization sequence, found immediately upstream of the bzip domain, causes export of C/EBPβ into the cytoplasm. Export is dependent on CRM1 and prevents expression of C/EBPβ target genes (Buck et al., 2001b). C/EBPβ can also be phosphorylated on a threonine residue by RSK. This modification causes the association

of phosphorylated C/EBPβ with procaspase 1 and 8, preventing caspase activation and apoptosis (Buck et al., 2001a).

The transcriptional activity of C/EBPβ can be further modulated by interaction with cofactor proteins. The association of C/EBPβ with the coactivator protein p300 and the chromatin remodeling protein Brahma enhances its transcriptional activation (Kowenz-Leutz and Leutz, 1999). C/EBPβ interacts with p300's E1A interacting domain via the first 21 amino acids of C/EBPβ, a motif not found in alternate translational starts (Mink et al., 1997). This interaction leads to increased transactivation by C/EBPβ and to phosphorylation of p300. The phosphorylation event causes redistribution of p300 within the cell nucleus where it colocalizes with C/EBPβ (Schwartz et al., 2003).

C/EBPδ is also expressed in the intestines, the lung and in adipose tissue. Its expression is upregulated by lipopolysaccharide and glucocorticoids. Although it is able to activate transcription as efficiently as other C/EBP family members, it binds DNA less efficiently due to sequence differences in the bzip region (Cao et al., 1991).

C/EBPα was the first C/EBP protein cloned (Landschulz et al., 1988; Umek et al., 1991). It is expressed in the liver, lung, intestine, placenta and adipose tissue. Due to its anti-mitotic actions, ectopic expression of C/EBPα causes cells to exit the cell cycle and thus, *in vivo* its expression is limited to terminally differentiated cells (Umek et al., 1991). For example, expression of C/EBPα is very low in proliferating hepatoma cells, but is much higher in mature hepatocytes (Friedman et al., 1989). Like C/EBPβ, multiple translational starts in the C/EBPα mRNA produces four isoforms with varying transcriptional activation potentials (Calkhoven et al., 2000).

Transcriptional control of preadipocyte differentiation

The process of preadipocyte differentiation is a highly coordinated cascade of transcriptional events that results in the development of the mature phenotype and in lipid accumulation (Fig. 3). The cascade is enabled when dividing preadipocytes reach confluence. Strongly contact inhibited, the achievement of confluence causes growth arrest of the cultures. Cell-cell contact appears to be an important signal for preadipocyte differentiation and leads to the expression of early markers such as lipoprotein lipase and type IV collagen (Dani et al., 1990). When post-confluent cells are treated with hormonal cocktail including insulin, a cyclic AMP phosphodiesterase isobutylmethylxanthine (MIX), and glucocorticoids they differentiate efficiently into mature adipocytes within 7-10 days (Fig. 3 bottom) (Green and Kehinde, 1975; Rubin et al., 1978).

Treatment with the hormonal cocktail causes the rapid induction of the early differentiation regulators C/EBPβ and δ (Fig. 3 top) (Cao et al., 1991). RNA transcripts for both C/EBPβ and δ are detectable immediately after treatment and remain detectable for up to five days. The proteins are maximally expressed 24 hrs after treatment. C/EBPβ levels remain high up to 48 hrs and then steadily decrease. C/EBPδ levels drop sharply by 4 days following treatment (Cao et al., 1991). Transcription by these two factors leads to the induction of factors involved in the development of the adipocyte phenotype, especially C/EBPα.

Despite upregulation of both C/EBPβ and δ protein levels, their transcriptional activity is restrained by heterodimerization with the inhibitory family member C/EBPζ/CHOP. C/EBPζ/CHOP is a bzip protein that has two proline residues in the DNA binding domain that impair DNA-binding, though it is still able to dimerize efficiently with C/EBP family members (Ron and Habener, 1992). Although the protein levels of CHOP begin to drop following the induction to differentiate, the levels are not significantly reduced enough before twelve hours post-induction to allow for C/EBPβ or δ-mediated transcription. After twelve hours, both

C/EBPβ and δ can be seen associated with DNA response elements in their target gene promoters (Tang and Lane, 2000).

Inactivation of C/EBPβ has little impact on the formation of white fat *in vivo* though deletion of both C/EBPβ and δ results in significantly reduced fat pad mass attributed to decreased adipocyte numbers. C/EBPβ and δ therefore play a role in committing cells to the adipocyte lineage (Tanaka et al., 1997).

Interestingly, when C/EBPβ is expressed from the C/EBPα locus *in vivo*, the mice demonstrate fat pads with the same number of differentiated cells as wild-type controls. However, C/EBPα has additional roles in promoting lipid accumulation as demonstrated by the reduced white adipose tissue mass attributed to decreased adipocyte size and the reduced expression of the adipocyte marker adipsin (Chen et al., 2000).

Recently, C/EBPβ has been implicated in the progression of the cell cycle. In fact the upregulation and acquisition of DNA binding by C/EBPβ correlates with the mitotic clonal expansion of induced preadipocytes, a process which is required for 3T3 L1 preadipocyte differentiation (Patel and Lane, 1999; Tang et al., 2003b). Mouse embryonic fibroblast lacking C/EBPβ expression do not undergo mitotic clonal expansion in response to differentiation stimulus and do not differentiate into adipocytes (Tang et al., 2003a). Restoration of C/EBPβ protein levels in these cells results in reentry into the cell cycle and the development of the mature adipocyte phenotype. The termination of mitotic clonal expansion and growth arrest correlates with the appearance of C/EBPα. C/EBPα is profoundly anti-mitotic and its expression in dividing cells causes cessation of the cell cycle and terminal differentiation (Umek et al., 1991).

The upregulation of C/EBPα is controlled by a C/EBP motif in its proximal promoter that is bound in early differentiation by C/EBPβ and C/EBPδ (Fig. 3 top) (Legraverend et al., 1993). Upregulation of C/EBPα protein allows C/EBPα to autoregulate its own transcription through

this element, thereby maintaining adipocyte gene expression during terminal differentiation (Christy et al., 1991; Legraverend et al., 1993). In fact, the appearance of C/EBPα corresponds to the downregulation of the early regulators C/EBPβ and δ (Cao et al., 1991). The importance of C/EBPα for the development of the adipocyte phenotye is demonstrated by genetic ablation of this protein in mice. Loss of C/EBPα expression leads to defective lipid storage with no accumulation of lipid in white adipose tissue. Antisense RNA directed against C/EBPα prevented the accumulation of triacylglycerol in 3T3 L1 cells (Lin and Lane, 1992). Conditional expression of C/EBPα triggers differentiation and the expression of adipocyte markers such as 422/aP2 (Lin and Lane, 1994).

One of the C/EBPα target genes is PPARγ, a member of the peroxisome proliferator activated receptor family (Rosen et al., 2002). Transcription is controlled by two consensus C/EBP motifs present in the proximal promoter, and other family members have been shown to bind this element (Elberg et al., 2000). PPARγ is the second major regulator that controls the terminal differentiation phase. PPARγ is able to auto- and cross-regulate its own promoter and the C/EBPα promoter (Wu et al., 1999). The appearance of C/EBPα and PPARγ is required for the activation of downstream adipocyte specific genes such as adipsin and glycerol-3-phosphate dehydrogenase (GPDH) (Fig. 3 top).

Experimental rationale

Boruk and coworkers identified a non-genomic mode of action for GR in which the ligand binding domain (LBD) is capable of enhancing C/EBPβ-mediated transcription from a synthetic C/EBP responsive promoter and from the herpes simplex thymidine kinase promoter (Boruk et al., 1998). The potentiation of transcription by GR was ligand dependent, independent of the ability of GR to bind DNA, and did not involve direct protein-protein interaction between

the GR LBD and C/EBPβ. The effect on transcription was also specific for C/EBPβ as it did not occur with other family members

Both glucocorticoids and C/EBPβ are important early regulators of the preadipocyte differentiation process. Preadipocyte differentiation of 3T3 L1 cells therefore provides an excellent model to study the physiological impact of the functional interaction demonstrated between the glucocorticoid receptor and C/EBPβ. Furthermore, the *in vivo* targets of C/EBP-mediated transcription, C/EBPα and PPARγ, can be used as tools to define the molecular mechanism of glucocorticoid action in this system.

The aim of this thesis was to elucidate the non-transcriptional mechanism of action for the glucocorticoid receptor during preadipocyte differentiation. My specific research goals were as follows. The initial goal of this project was to validate the preadipocyte differentiation system for the study of the GR-C/EBPβ functional interaction. To do so, the GR ligand binding domain was expressed through retroviral infection into preadipocytes and the effect of dexamethasone was evaluated. Second, to establish the sufficiency of the receptor ligand binding domain to transduce the effects of steroid in this system, a differentiation systen expressing the progesterone receptor ligand binding domain was used. The progesterone receptor, highly similar to the glucocorticoid receptor, is not expressed in 3T3 L1 cells. Activation of the progesterone receptor ligand binding domain by progestins would not activate the endogenous GR and could provide a system where the sufficiency of the LBD could be tested.

Once the cell culture model was validated, the next goal was to identify a target gene to study the molecular mechanism involved in the potentiation of preadipocyte differentiation by the GR ligand binding domain. Using the C/EBPα gene as a model, HDAC1 was identified as a repressor of C/EBPβ action.

Subsequent to the identification of a deacetylase as a repressor protein in this system, the project goals were expanded. As histone deacetylase proteins often function as part of large

multi-protein complexes, the first aim was to identify the complex, by coimmunoprecipitation and by FPLC, that was responsible for repressing C/EBPβ-mediated transcription of the C/EBPα promoter. Secondly, the role of GR ligand binding domain in relieving HDAC1-mediated repression was investigated using coimmunoprecipitation and Western analysis.

Since the transcriptional activity of C/EBPβ was found to be repressed by HDAC1, it was hypothesized that C/EBPβ could be a target for acetylation. The acetylation of C/EBPβ was mapped and the *in vivo* effects of acetylation were studied by expressing acetylation mutant proteins in the preadipocyte differentiation cell culture model. By coimmunoprecipitation and *in vitro* acetylation experiments the relevant acetylases were identified and their interactions with C/EBPβ were established.

MATERIALS AND METHODS

Expression Constructs

Clones employed are shown in Appendix A, tables 1 through 5 and are organized according to their source or cloning strategy. Table 1 lists clones obtained from other researchers. Common cloning vectors are listed in Table 2.

All plasmids were constructed using routine molecular cloning techniques. Where possible, inserts were cut from existing plasmids and inserted into the desired vector using convenient restriction sites (Appendix A, Table 3). Otherwise, inserts were PCR amplified with primers containing the desired restriction sites and inserted into the appropriate vector (Appendix A, Table 4).

For site directed mutagenesis of existing plasmids, Stratagene's PCR mutagenesis kit® was used (Appendix A, Table 5). Briefly, primers were designed for both DNA strands containing the desired mutation and 5' and 3' complementary sequence. To amplify the plasmid with the polymerase Pfu, 50ng of each primer was used. The PCR products were then digested for 1 hr with DpnI, which cleaves methylated DNA, to digest away the reaction template. The remaining PCR product was then transformed by electroporation into competent bacteria.

In all cases, clones were screened using restriction enzyme digests to confirm the insert orientation followed by DNA sequencing.

Bacterial Culture and Plasmid Preparation

Competent *Escherichia coli* DH5α strain were transformed with plasmid DNA by electroporation and plated on agar plates containing 100mg/ml of ampicillin. All reporter constructs were transformed into *Escherichia coli* RB404 strain (*dam*$^-$ *dcm*$^-$) to prevent the formation of cryptic glucocorticoid response elements by methylation of BamHI restriction sites

(Truss et al., 1992). Single colonies were chosen to inoculate large-scale cultures (500 ml- 1 L). Plasmids were purified using standard alkaline lysis protocols (Sambrook, 1989). This purification was followed by two consecutive cesium chloride gradients and dialysis.

Transient Transfection Analysis of Reporter Gene Expression

Cell Maintenance

Cos7 and HeLa cells (ATCC) were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with non-essential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS). Phoenix Ampho cells (ATCC, with permission from Dr. G. Nolan) were maintained in DMEM supplemented with penicillin, streptomycin and 10% FBS. Cells of the NIH 3T3 line were grown in DMEM supplemented with penicillin, streptomycin and 10% calf serum (CS). All of the cell lines were grown at 37 °C in a humidified atmosphere of 5% carbon dioxide. Cells of the 3T3 L1 line cells were maintained at 10% CO_2 in low glucose DMEM (1.5 g glucose per litre) supplemented with penicillin, streptomycin and 10% CS.

Transient Transfection

For transfection Cos7, HeLa and NIH 3T3 cells were seeded at a density of 4.0×10^5 cells per 60 mm tissue culture dish. Cells were transfected the following day. Cos7 cells were transfected using the Lipofectamine reagent. DNA (50-400 ng as indicated in figure legends) was diluted into 200 µl OptiMem media and complexed with 10 µl Lipofectamine prediluted into 200 µl OptiMem for a minimum of 1 hr at room temperature. This combination provides sufficient DNA for duplicate plates. The cells were washed twice with PBS and 2 ml of OptiMem were added prior to transfection. Complexed DNA (200 µl) was added to each plate. Cells were returned to the incubator for 16 hrs and the reaction was stopped by adding 2 ml of

phenol red free DMEM supplemented with non-essential amino acids, penicillin, streptomycin, and 20% charcoal stripped FBS (SFBS). For hormone treatments, 24 hrs following transfection, cells were treated with 10^{-6} M hormone agonist or antagonist, vehicle, 160 nM histone deacetylase inhibitor trichostatin A (TSA), and 1 μM of the proteasome inhibitor MG132 for 24 hrs.

HeLa and NIH 3T3 cells were transfected using ExGen 500. DNA for duplicates was diluted in 100 μl 150 mM NaCl and combined with 100 μl of 8 μl/μg DNA ExGen diluted in NaCl. The DNA-ExGen mixture was incubated at room temperature for 10 min and 100 ml of the mixture was added to the 2 ml of serum free media on each duplicate plate. The plates were washed twice with PBS and once with phenol red free DMEM prior to transfection. The plates were incubated for 3 hr at 37 °C and the transfections were stopped with DMEM containing 20% SFBS. Hormone treatments were as for Cos7 cells.

β-galactosidase assay

Cells were washed twice in PBS and 400 ml of 1X Reporter Lysis Buffer was added to each plate. Cells were scraped and collected in microfuge tubes. Extracts were centrifuged briefly to pellet cellular debris and supernatants were transferred into fresh tubes. For β-galactosidase assays, 100 μl of extract was mixed with 100 μl of Z buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mMMg_2SO_4, 50 mM β-mercaptoethanol). The reactions were started by the addition of 42 μl of 4 mg/ml of o-nitrophenyl β-D-galactopyranoside and incubation at 30 °C. The reaction was carefully timed using a stopwatch and upon development of a yellow colour, the reactions were rapidly stopped by addition of 100 μl of 1 M Na_2CO_3. Optical density of the samples was measured at λ=420 nm and was used to calculate β-galactosidase activity using the following formula: β-galactosidase units/ml = $OD_{420} \div$ (0.0045 X reaction time (min) X volume of extract (ml)). All data are shown as means and error bars

represent the standard error of the mean for a minimum of three independent experiments performed in duplicate.

Luciferase assay

A portion of the extract (20 μl) used in the β-galactosidase assay was placed in 12 X 75 mm borosilicate glass tubes. Using the auto-inject device of the Monolight 2010 Luminometer (Applied Luminescence Laboratory), 100 ml of Luciferase Assay Reagent was mixed with the extract and the light produced (in relative light units) was measured for 10 s. Light intensity is proportional to luciferase concentration in the range of 10^{-16} M (10 ρg/L) to 10^{-8} M (1 mg/L). All data are shown as means and error bars represent the standard error of the mean for a minimum of three independent experiments performed in duplicate.

Retroviral Infection

Transfection of Phoenix Ampho Cells

Replication incompetent pLXSN-based (Clontech) retroviruses were generated by transfection of Phoenix Ampho (ATCC) packaging cells. Phoenix cells were seeded at a density of 1.0 X 10^6 cells per 60 mm dish. The following day cells were transfected with 10 μg of the retroviral vector by calcium phosphate precipitation. The DNA was diluted in 432 ml pyrogen free water and 62 ml of 2M $CaCl_2$ was added. To this mixture, 500 ml of 2X HBS (50 mM HEPES pH 7.05, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added, the tube was mixed by tapping and the mixture was immediately added to the Phoenix cells in 4 ml of media containing 25 μM chloroquine. The cells were returned to the incubator for 10 hrs and then the media was changed. The following day the media was changed again and the cells

were incubated for an additional 24 hrs after which the viral supernatants were collected and filtered through a 40 μm filter.

Infection of 3T3 L1 and NIH 3T3 cells

Dishes (10 cm) of 50% confluent 3T3 L1 cells were infected overnight using 1ml of viral supernatant mixed with 3 ml of media containing 4 μg/μl polybrene. The infection was stopped by adding 6 ml of media to the plate. After 48 hr of infection the cells were selected in media containing 400 μg/μl G418 for 10 days prior to differentiation to ensure expression in all cells.

Differentiation of 3T3 L1 Preadipocytes

3T3 L1 cells were maintained in DMEM containing 1.0 g L^{-1} glucose supplemented with 10% CS in 10% CO_2. Calf serum lots were carefully type-matched to provide consistent responses to hormonal cocktail. To stimulate differentiation, 2-day post-confluent preadipocytes (designated day 0) were treated with 50-100 nM insulin, 500 μM 3-isobutyl-1-methyl-xanthine (MIX) and 250 nM dex, 250 nM of the synthetic progestin R5020, 400 nM TSA or 10 mM valproic acid (VPA) for 48 hours as indicated in figure legends. Cells were subsequently re-fed DMEM supplemented with 10% CS and insulin every other day for a total of 8 days. Phase contrast photomicrographs are representative of a minimum of 3 experiments performed in duplicate.

Glycerol-3-Phosphate Dehydrogenase Assay

Differentiated 3T3 L1 cells in 6 well dishes were washed twice in ice-cold PBS and harvested in 250 ml of Tris-EDTA-2ME buffer (25mM Tris pH7.4, 1mM EDTA, 1mM 2-mercaptoethanol). The cell suspension was vortexed and sonicated for 5 s at a duty cycle of 30%

(Branson sonifier model-450, Branson Ultrasonics, Markham, Ontario). Samples were centrifuged for 10 min at 95 000 rpm (rotor TLA-120.1, centrifuge Optima-MAX, Beckman Coulter, Mississauga, Ontario) at 4°C and the supernatants were transferred to fresh tubes. In quartz cuvettes, 50 µl of the sample was mixed with 50 µl of the Tris-EDTA-2-ME buffer, and 60 µl GPDH cocktail (346 mM Triethanolamine pH 7.7, 167 mM 2-mercaptoethanol, 0.8 mM EDTA, 2 mg β-NADH). The reaction was started by the addition of 60 µl of a 4 mM solution of the substrate, dihydroxyacetone phosphate, and the absorbance at λ=340 was measured every 10 s for 2 min. The GPDH activity was calculated as follows: $((A_{t=0}-A_{t=2})$ X 32.154) / 50 µl X 1000 µl/ml = unit/ml/min (Wise and Green, 1979). These activities were corrected for total protein using a standard Bradford assay. Error bars represent the standard error of the mean for three independent experiments.

Oil Red O Staining

Cells were gently washed twice with PBS and fixed by incubation at room temperature with 2 ml of 10% formalin in PBS for 1 hr. Cells were then washed once with PBS and stained with 2 ml of Oil Red O (3.5g in 500 ml propylene glycol) for 2 hr to overnight. Cells were then washed extensively with distilled water to remove excess stain (Schwarz et al., 1997).

Immunoprecipitation and Western Analysis

Preparation of whole cell extracts

Plates (10 cm) were washed once with PBS and scraped in 500 µl PBS into microcentrifuge tubes. Cells were pelleted by centrifugation at 4°C for 5 min at 4000 X g. For coimmunoprecipitation experiments cells were resuspended in 100 ml of TEDG buffer (10 mM

Tris pH 7.4, 1 mM EDTA, 10% glycerol, 1 mM 1,4-dithiothreitol (DTT), 1X complete protease inhibitor cocktail) and allowed to swell to 10 min on ice. Extracts were produced using three cycles of freezing in methanol and dry ice followed by thawing at 37°C and vortexing. For immunoprecipitation or direct Western analysis cell pellets were resuspended in 100 µl of IPH buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM DTT, 1X complete protease inhibitor cocktail). Cells were allowed to swell on ice for 10 min and were then sonicated for 10 s at a 30% duty cycle. In both cases, cell extracts were centrifuged at 13 000 X g for 5 min at 4°C to pellet cellular debris and the supernatants were transferred to fresh tubes. Protein concentrations were determined by a standard Bradford assay.

Preparation of Protein A Sepharose

Protein A immobilized Sepharose beads were hydrated overnight in distilled water. The following day, the beads were blocked overnight in IP buffer (50 mM Tris pH7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Nonindet P40 (NP-40), 0.02% sodium azide) supplemented with 50 mg/ml of bovine serum albumin. The beads were then stored as a 50% slurry in IP buffer.

Crosslinking Antibody to Protein A Sepharose beads for C/EBPβ immunoprecipitation

To allow the antibody to bind, approximately 1 mg/ml of anti-C/EBPβ antibody or non-specific type matched antibody was incubated with the hydrated protein A sepharose beads at room temperature with gentle rocking. The beads were then washed twice in 10 volumes of 0.2 M sodium borate pH 9.0 by centrifugation at 3500 X g for 5 min. The beads were then resuspended in 10 volumes of sodium borate and dimethylpimedilate was added to a final concentration of 20 mM. The beads were incubated for 30 min at room temperature and the reaction was stopped by washing the beads twice with 0.2 M ethanolamine. Beads were then

incubated for 2 hrs in 10 volumes of ethanolamine with gentle rocking. After this final wash, the beads were washed once in 100 mM glycine pH 3.0 to remove any uncoupled antibody from the beads. The beads were then stored as a 50% slurry in PBS containing 0.01% thimerosal as a preservative.

Immunoprecipitation and coimmunoprecipitation

Cellular extracts (500 –1000 μg) were diluted in 1X binding buffer (25 mM HEPES pH7.9, 50 mM KCl, 0.5 mM EDTA, 12% glycerol, 0.1% NP-40, 0.2 mM DTT, 1X complete protease inhibitor cocktail) to which 50 μl of the coupled beads (approximately 10 μg antibody) was added and allowed to incubate at 4°C with rotation for 2 hrs. Immunoprecipitates were washed twice by collecting the beads after centrifugation at 4000 X g for 2 min at 4°C, and gently adding 1 ml of wash buffer (50 mM Tris pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1% Triton X-100). The precipitates were mixed by inversion. After the final wash the immunoprecipitates were resuspended in SDS-PAGE loading buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 0.05% bromophenol blue, 355 mM 2-mercaptoethanol).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out according to standard protocols. Protein samples in SDS loading buffer were boiled for 5 minutes and centrifuged at 13 000 X g for 2 minutes prior to loading. Samples were loaded on a 6-10% mini protein gel with a 1 cm 4% stacking gel and separated according to the desired resolution. Gels used for Western blot were transferred onto PVDF membrane using standard procedure.

Western Analysis

PVDF membranes (Bio-Rad, Mississauga, Ontario) were rinsed briefly in PBS-T (PBS with 0.5% Tween 2) and blocked in PBS-T + 5% skim milk for 1 hr at room temperature. Membranes were then incubated with a PBS-T/primary antibody solution for 2 hrs at room temperature or overnight at 4°C. The primary antibodies were used at the following concentrations: C/EBPβ C-19, HDAC2 C-8, mSin3A AK-11, RbAp48 N-19, RbAp46 N-19, GR M-20, PR C-19, GCN5 N-18, PCAF H-369 (1:400 dilution, all from Santa Cruz Biotech, Santa Cruz, California), HDAC1 (1:1000, Affinity Bioreagents, Golden, Colorado), p300 (1:1000, Upstate Biotech, Charlottesville, Virginia) or a pan acetyl lysine polyclonal antibody ab193 (1:1000, Abcam, Cambridge, Massachusetts). Membranes were washed extensively with PBS-T and incubated with horseradish peroxidase conjugated secondary antibodies (anti-rabbit Fab fragments 1:10 000, anti-mouse 1:50 000, Amersham, Baie D'Urfe, Quebec, anti-goat 1:10 000, Santa Cruz, Santa Cruz, California). Signals were detected by chemiluminescence (Western Lightning, Perkin Elmer, Woodbridge, Ontario).

GST pulldown assay

Expression of GST fusion proteins in bacteria

GST fusion expression plasmids were transformed by heat shock into competent *Escherichia coli* BL21DE3 (pLys) strain, and single colonies were chosen for overnight culture. This culture was used to inoculate a larger scale culture (50-500ml) at a 1:100 dilution. Cultures were allowed to grow with shaking at 37 °C until the absorbance at λ=600 nm reached 0.6-0.8. Bacteria were then induced by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 2 hrs. Cells were collected by centrifugation (4000 X g, 10 min, RT) and resuspended in lysis buffer (750 μl for a 50 ml culture, 25 mM HEPES pH7.9, 100mM KCl, 2

mM EDTA, 20% glycerol, 2mM DTT, 1x complete protease inhibitor cocktail). NP-40 was added to a final concentration of 0.1% and suspensions were sonicated at a 35% duty cycle, and centrifuged to collect cellular debris (13 000 X g, 10 min, 4°C). The supernatants were transferred to fresh tubes and incubated for 1 hr at 4°C with rotation with glutathione sepharose beads that were extensively washed in lysis buffer + 0.1% NP-40. Following incubation with the beads the precipitates were washed three times with 1 ml of lysis buffer. Coomassie stained polyacrylamide gels were used to quantify GST fusion proteins by comparing their expression with bovine serum albumin standards proteins.

In vitro Translation of Protein

In vitro translation of proteins was accomplished using the TNT® T7 Coupled Reticulocyte Lysate System (Promega, Madison, Wisconsin) according to manufacturer's instructions. Proteins were resolved by SDS-PAGE and stained with Coomassie blue. Phosphorimager analysis (Typhoon, Molecular Dynamics (Amersham Biosciences), Baie D'Urfe, Quebec) was used for relative quantification of proteins for use in binding assays.

Binding assay

To analyze binding to C/EBPβ *in vitro*, 1 μg GST or GST-C/EBPβ was incubated with *in vitro* translated ^{35}S-labelled mSin3A or HDAC1 by incubation for 2 hr in 0.6X lysis buffer +0.1% NP-40. Following extensive washings in 0.6X lysis buffer + 0.1% NP-40, precipitates were resolved by SDS-PAGE and dried gels were visualized by phosphorimager analysis. Binding was compared to 10% input of the radiolabelled protein.

Northern Analysis of HDAC1 mRNA

Total RNA from 3T3 L1 cells was obtained using the RNeasy® RNA Isolation kit (Qiagen, Mississauga, Ontario). RNA (10 μg) was separated on a 1.0% denaturing agarose gel and transferred to nitrocellulose membrane (Amersham Biosciences, Baie D'Urfe, Quebec) by capillary action. HDAC1 mRNA was detected using a 311 base pair ^{32}P-labelled probe derived from human HDAC1 (amino acids 321-425).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitations were performed essentially as described (Yahata et al., 2001). Cells of the 3T3 L1 line were treated with vehicle, MIX/insulin or MIX/insulin and dex in the presence or absence of 1 μM MG132 as indicated for 24 hrs and cells were washed 2X in serum free media and treated with 1% formaldehyde at room temperature for 10 minutes. After harvesting and washing in PBS and in buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA and 10 mM Hepes, pH 6.5) and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES pH6.5) cell pellets were resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH8.0, 1 mM DTT, 1x complete protease inhibitor cocktail) and sonicated three times. Extracts were centrifuged (13,000 g, 10 min, 4° C) and supernatants were diluted 20X in dilution buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 50 mM Tris pH8.0, 1mM DTT, 1x complete protease inhibitor cocktail) and incubated with antibodies to gal4dbd (non-specific antibody), C/EBPβ C-19, mSin3A AK-11, HDAC1 C-19, p300 N-15, RNA polII N-20, GCN5 H-75 (all from Santa Cruz Biotech, Santa Cruz, California) and acetyl-H4 (Upstate Biotechnology, Charlottesville, Virginia) as specified at 4° C overnight. Precipitation was accomplished using a protein A-sepharose slurry with 2μg of sheared salmon sperm DNA. Precipitates were then washed sequentially for 10 minutes each at 4° C in TSE I (0.1% SDS, 1%

Triton X-100, 2 mM EDTA, 20 mM Tris pH8.0, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton x-100, 2 mM EDTA, 20 mM Tris pH8.0, 500 mM NaCl), buffer III (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate (EM Science), 1 mM EDTA, 10 mM Tris pH 8.0) and twice in TE and extracted 3X in 100µl of 1% SDS, 0.1 M $NaHCO_3$. Eluates were heated to 65° C overnight to reverse cross-links. DNA fragments were purified using the Qiaquick PCR purification kit™ (Qiagen, Mississauga, Ontario) and amplified by PCR using the following primers for positions within the murine C/EBPα promoter: -334 and –118 (5'-TAGTGTTGGCTGGAAGTGGGTG ACTTAG AGGC-3', 5'-TTCTCCTGTGACTTTCCAAGGCGGTGAGTG-3'), -108 and +17 (5'-TAAGACC CAGCAGGCACCAT CCTACTG-3', 5'-AGTTAGAGTTCTCCCGGCATGG CGAG-3'). Results shown are representative of a minimum of 3 independent experiments.

Chromatographic separation of HDAC1 containing complexes

Extracts were prepared from HeLa or 3T3 L1 cells 24 hrs post-induction of differentiation with MIX/Insulin or MIX/Insulin/dex in TEDG. Extract (1-2 mg) was separated by FPLC using a Sephacryl S300 HR column and TEDG as the elution buffer. Fractions were collected and precipitated in 15% trichloroacettic acid at -20° C for 30 minutes. Precipitates were washed once in ice-cold acetone and resuspended in SDS loading buffer. Precipitates were resolved by SDS-PAGE and membranes were probed with the indicated antibodies (see section of Western analysis for antibodies).

Detection of *in vivo* acetylation

Cell extracts were performed as described and 5 mg of extracts were immunoprecipitated with coupled anti-C/EBPβ protein A sepharose beads for 2 hrs at 4°C. Precipitates were washed three times with wash buffer and resolved on a 10% SDS-PAGE. All buffers contained 5 µM

TSA to inhibit deacetylation during handling and blots were probed with the ab193 antibody (abcam, Cambridge, Massachusetts).

Production of recombinant GCN5

His-GCN5 expression plasmid was transformed by heat shock into competent *Escherichia coli* BL21DE3 (pLys) strain, and single colonies were chosen for overnight culture. Large scale cultures (50 ml) were produced by diluting cultures 1:100 and allowing bacteria to divide until an absorbance at l=600 nm of 0.6-0.8 was achieved. Cells were pelleted by centrifugation at 4000 X g and recombinant GCN5 was purified under native conditions using the Ni-NTA Spin kit (Qiagen, Mississauga, Ontario).

***In vitro* acetylation**

Equal amounts of GST fusion proteins (5-10 μg) and core histones (10 μg) were incubated with 500 ng of recombinant PCAF, recombinant GCN5 or recombinant p300 in 1X HAT buffer (50 mM tris pH 8.0, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) with 0.2 μCi of ^{14}C-acetyl CoA at 30 °C for 1 hr. SDS loading buffer was added to the reactions and samples were resolved by 15% SDS-PAGE. Samples were transferred to PVDF membrane and probed for the modification using the ab193 antibody. Membranes were then dried and radiolabelled proteins were analyzed by phosphorimager.

RESULTS

The Glucocorticoid receptor ligand binding domain can potentiate transcription mediated by C/EBPβ.

C/EBPβ is a well-characterized transcription factor that acts in several pathways occurring during cell differentiation such as liver regeneration, the development of immune responses and adipogenesis. In all of these processes, C/EBPβ acts in part by activating the transcription of commitment factors, in particular C/EBPα. The upregulation of C/EBPα by C/EBPβ has been demonstrated in hepatocytes (Diehl, 1998) and exists in preadipocytes (Cao et al., 1991), though it can be masked by cross-regulation between C/EBPα and PPARγ (Wu et al., 1999). C/EBPα and PPARγ were independently shown to be necessary and sufficient for the preadipocyte differentiation process (Lin and Lane, 1994; Tontonoz et al., 1994), and their genes are regulated at the transcription level by one or more C/EBP motifs in the enhancer regions. The C/EBPα minimal promoter is controlled by a centrally located C/EBP motif that is highly degenerate (Fig. 4A), but has been shown to bind C/EBPα, β and δ by electrophoretic mobility shift assay (Legraverend et al., 1993). The PPARγ promoter is also bound by these C/EBP family members to two near consensus sites (Fig. 5A) (Elberg et al., 2000).

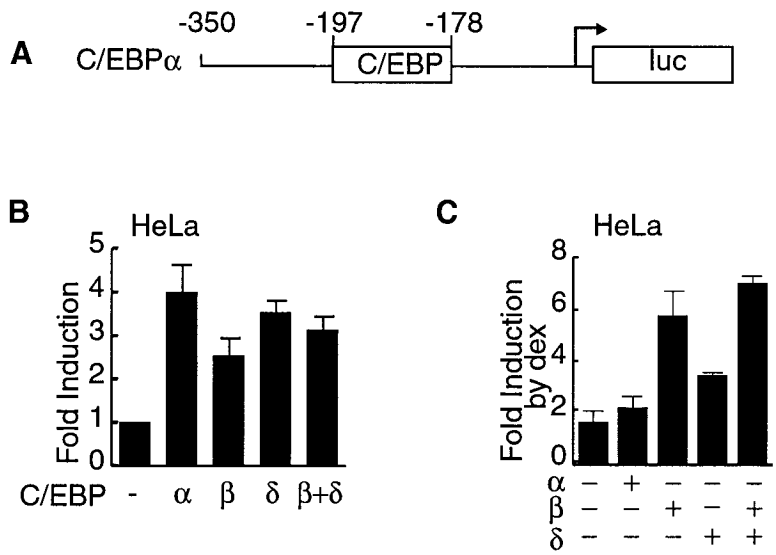
In transient transfection assays in HeLa cells, both C/EBPα and C/EBPδ were able to activate transcription from the C/EBPα promoter by approximately 3.5-fold as measured by luciferase production (Fig. 4B). C/EBPβ was also able to activate transcription from this promoter, but to a lesser extent (2.2-fold). In similar experiments, in which the transfected cells were treated with 10^{-6} M dexamethasone for 24 hours before harvesting to activate the endogenous glucocorticoid receptor, it was observed that steroid treatment potentiated transcription from the C/EBPα promoter 6-fold in the presence of C/EBPβ (Fig. 4C). The

Figure 4. The glucocorticoid receptor ligand binding domain enhances C/EBPβ mediated transcription from the C/EBPα promoter.

(A) Schematic representation of the murine C/EBPα proximal (-350/+7) promoter used in transient transcription experiments. The location of the C/EBP motif is indicated.

(B) Transactivation of the C/EBPα promoter by C/EBPα β, δ, and the βδ heterodimer in HeLa cells. 60 mm dishes of HeLa cells were transfected with 200 ng of the C/EBPα reporter (pCX14/12) and 400 ng of the pMSVC/EBP expression plasmids. In the case of the βδ heterodimer, 200 ng of each expression plasmid was transfected. Promoter activity was determined through measurement of luciferase activity 48 hrs after transfection. Cotransfected βgal reporter plasmid (100 ng pRSV-βgal) was used to correct luciferase activity data for transfection efficiency by measuring the βgal activity in cell extracts. Data from a minimum of three independent experiments performed in duplicate is represented as fold induction over basal promoter activity. Error bars represent the standard error of the mean.

(C) Transcription by C/EBPβ and the βδ heterodimer is enhanced by dex treatment. HeLa cells were transfected as in (B) and treated 24 hrs after transfection for 24 hrs with 10^{-6} M dex. Transcription was measured as in (B) and is represented as fold induction by dex treatment over the level attained in the absence of dex.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C/EBPα
C/EBP
luc
B
HeLa
Fold Induction
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3
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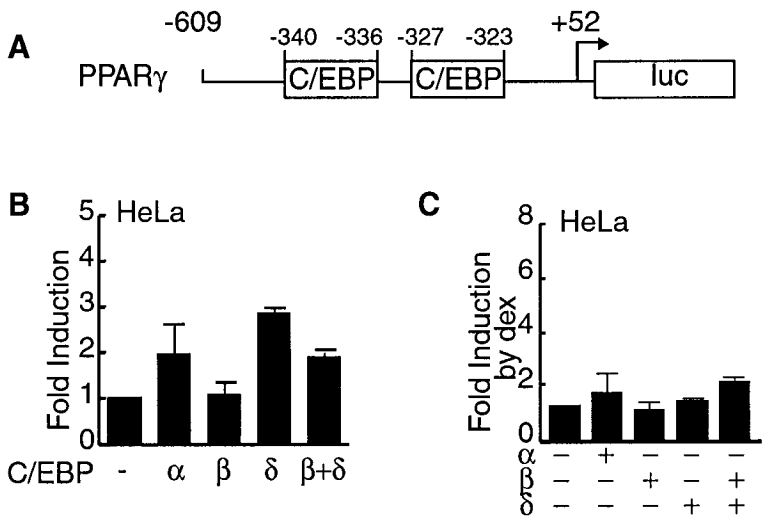
C/EBP - α β δ β+δ
C
HeLa
Fold Induction by dex
8
6
4
2
0
α – + – – –
β – – + – +
δ – – – + +

Figure 5. Transcription from the murine PPARγ promoter is not activated by C/EBPβ or potentiated by the glucocorticoid receptor.

(A) Schematic representation of the murine PPARγ proximal (-609/+52) promoter used in transient transcription experiments. The locations of the two conscensus C/EBP motifs are indicated.

(B) Transactivation of the PPARγ promoter by C/EBPα, β, δ, and the βδ heterodimer in HeLa cells. Dishes (60 mm) of HeLa cells were transfected with 100ng pRSV-βgal, 200ng of the PPARγ reporter (pC478) and 400ng of the pMSVC/EBP expression plasmids. In the case of the βδ heterodimer, 200ng of each expression plasmid were transfected. Transcription was measured by a standard luciferase activity and was corrected for transfection efficiency as measured by βgal activity.

(C) Transcription by C/EBPβ and the βδ heterodimer is not enhanced by dex treatment. HeLa cells were transfected as in (B) and treated for 24 hrs with 10^{-6} M dex. Transcription was measured as in (B).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-336
-327
-323
+52
PPARγ
C/EBP
C/EBP
luc
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HeLa
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C/EBP
-
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β
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β+δ
C
HeLa
Fold Induction by dex
8
6
4
2
0
α
β
δ

C/EBPβδ heterodimer was equally potentiated by steroid treatment above the level induced by C/EBPβδ (approximately 7-fold), while activation by C/EBPδ alone was minimally affected (2.5-fold). Treatment with glucocorticoids did not potentiate transcription by C/EBPα (Fig. 4C).

In contrast, C/EBPβ was unable under the same transfection conditions to activate transcription from the PPARγ promoter although family members C/EBPα and C/EBPδ activated transcription by 2 to 3-fold (Fig. 5B). This result is consistent with previously published work that showed that although C/EBPβ was able to bind consensus DNA motifs, it was unable to activate PPARγ transcription (Elberg et al., 2000). Activation of transcription by the other family members ranged from 2-fold for C/EBPα to 3-fold for C/EBPδ (Fig. 5B). Furthermore, as the βδ heterodimer activates transcription from the PPARγ promoter less efficiently (2-fold) than C/EBPδ alone (3-fold), this data suggests that C/EBPβ actually interfered with the activation of PPARγ by C/EBPδ. Steroid treatment did not result in a potentiation of transcription from the PPARγ promoter by any of the C/EBP family members (Fig. 5C).

Glucocorticoid potentiation of C/EBPα transcription by C/EBPβ was dependent on the presence of agonist, and was not observed upon treatment with the glucocorticoid antagonist RU486 (Fig. 6A). Treatment with dexamethasone resulted in a 6-fold increase in luciferase activity whereas treatment with the antagonist was without effect. Moreover, both the N-terminal activation domain and the DNA binding domain of the receptor were dispensable for potentiation of transcription. The full length GR mutant GR_{L501P}, which bears a point mutation in the DNA-binding domain which abrogates sequence specific DNA binding, and the truncated receptor bearing amino acids 505-795 of the rat receptor were both able to activate transcription in Cos7 cells which lack endogenous receptor (Fig. 6B and C). Expression of the wild type full-length receptor, the DNA binding point mutant or the ligand binding domain alone resulted in potentiation of C/EBPβ-mediated transcription from the C/EBPα promoter by 6 to 11-fold. The

Figure 6. The potentiation of C/EBPα transcription by the GR ligand binding domain requires DNA-bound C/EBPβ.

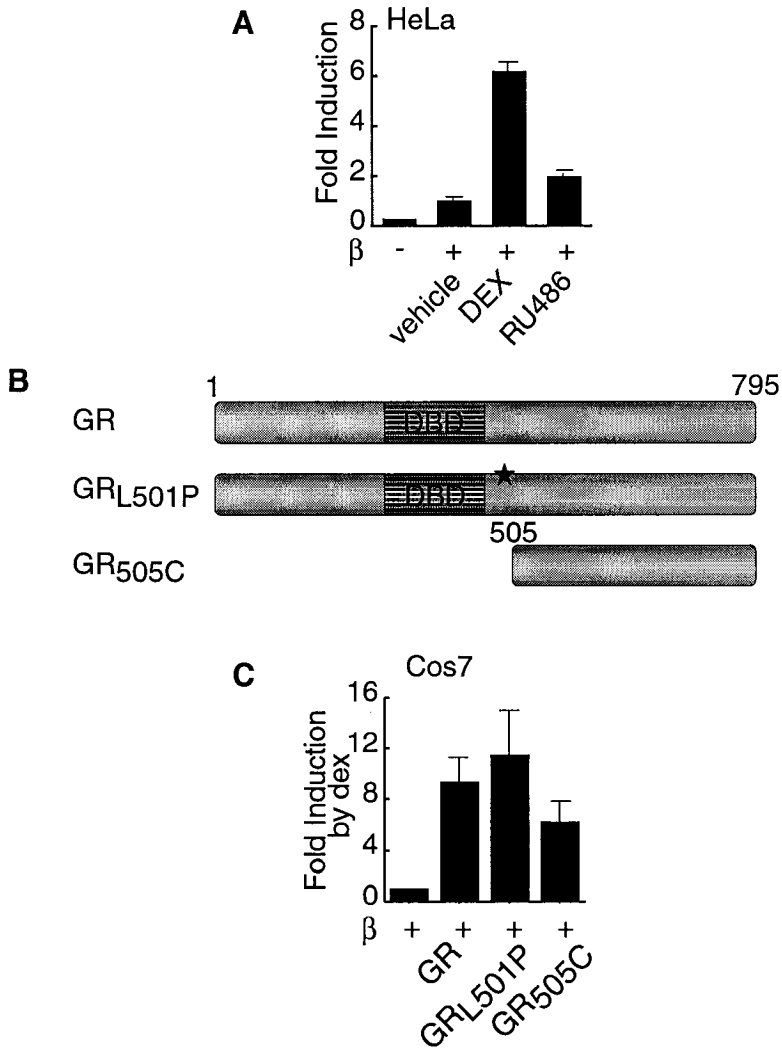
(A) HeLa cells were transfected as in Fig. 1 with pRSVβgal, the C/EBPα reporter and expression plasmid for C/EBPβ as indicated. Twenty-four hours post-transfection cells were treated for 24 hrs with vehicle (ethanol), 10^{-6}M dex or 10^{-6} M RU486. Data is represented as fold induction of activity by steroid over treatment with vehicle alone.

(B) Schematic representation of the GR constructs used in transient transfection experiments. GR_{L501P} has a point mutation at position 501 that abolishes DNA binding. GR_{505C} is a truncated receptor expressing only the C-terminal portion of the receptor from amino acids 505-795.

(C) The effect of dex on C/EBPβ transcription is mediated through the LBD alone. Cos7 cells were transfected with 100ng βgal, 200ng pCX14/12, 400ng of pMSVC/EBPβ and 100ng of full-length rat GR (pTLrGR), the DNA binding point mutant GR_{L501P}, or the LBD alone (GR_{505C}) as indicated. Transfected cells were treated for 24 hrs with dex and harvested for luciferase activity as before. Data is represented as fold induction by dex treatment.

(D) Sequence of the C/EBP motif of the murine C/EBPα promoter (-197 to –178) used in transient transfections. The mutation that abolishes C/EBP transactivation (C/EBPα mt) is also shown.

(E) The effect of dex is transduced through the C/EBP binding motif in the C/EBPα promoter. Cos7 cells were transfected with βgal, and pMSVC/EBPβ, and GR_{505C} as in (B) along with 200ng of the wild type reporter or the C/EBP site mutant as indicated. Data is shown as fold induction over the activity of the C/EBP site mutant.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vehicle
DEX
RU486
B
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795
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DBD
GRL501P
505
GR505C
C
Cos7
Fold Induction by dex
16
12
8
4
0
β + + + +
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GRL501P
GR505C
D
-197
-178
C/EBPα GTCAGTGGGCGTTGCGCCACGATCTCT
C/EBPα mt GTCAtTcGcacagGtagCtCGAgtTCT
E
Cos7
Fold Induction
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10
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0
β + + + +
GR505C - + - +
wt
mt
C/EBPα

difference in potentiation of transcription by the LBD, though not statistically different from the full length constructs may be due to decreased protein stability upon expression. These results suggest that direct contact with the DNA is not required for transcriptional activation by the receptor, and further that the receptor acts through a non-genomic or non-transcriptional mechanism. The potentiation of transcription by the GR ligand binding domain was however dependent on DNA bound C/EBPβ, as site-directed mutation of the C/EBP motif (a 19 base pair substitution, see Appendix I, table 5) within the C/EBPα promoter (Fig. 6D) abolished transcription from this promoter, and eliminated the effect of the GR LBD (Fig. 6E). Whereas C/EBPβ was able to activate transcription from the C/EBPα promoter by approximately 3-fold, it was completely ineffective in activating transcription from the mutated promoter. Coexpression of the GR ligand binding domain (GR_{505C}) and treatment with agonist enhanced C/EBPβ mediated transcription by 9-fold on the wild type promoter, but had no effect on the mutant promoter, highlighting the requirement for DNA-bound C/EBPβ.

Ectopic expression of the GR ligand binding domain can potentiate the effects of steroid in preadipocyte differentiation.

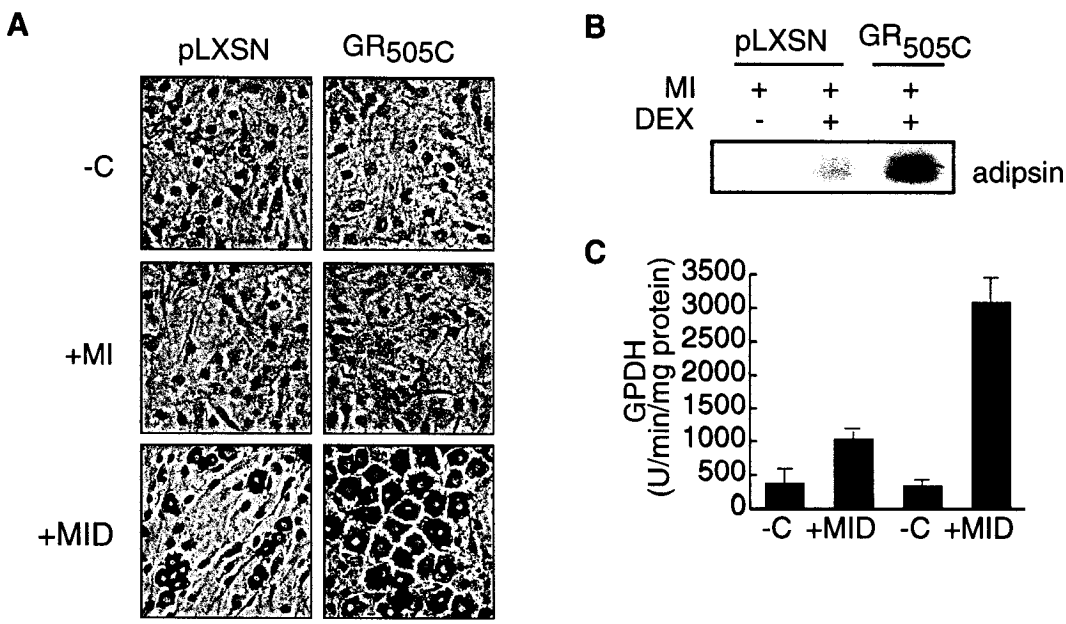
Since both C/EBPβ and GR are important early regulators in the process of preadipocyte differentiation, it was decided to determine if the GR ligand binding domain was able to influence C/EBPβ-mediated transcription *in vivo*, and consequently the differentiation process. Retroviral expression of the GR ligand binding domain (GR_{505C}) in 3T3 L1 preadipocytes potentiated their differentiation in the presence of dex treatment when compared to cells infected with empty virus (pLXSN). Dex treatment in the presence of the GR ligand binding domain (GR_{505C}) increased the number of cells staining with Oil red O by approximately 3-fold (Fig. 7A), and increased the expression of the adipocyte marker protein adipsin by approximately 3-fold (Fig. 7B). In the absence of treatment with differentiation cocktail (-C), cells expressing the GR ligand binding

Figure 7. The GR LBD can potentiate adipogenesis

(A) Oil Red O staining of 3T3 L1 cells infected with control virus (pLXSN) or with virus expressing the GR LBD (GR_{505C}) 8 days after treatment with MIX and insulin (+MI), MIX, insulin and dex (+MID) or in the absence of cocktail (-C) for the first 48hrs of the differentiation protocol. Cells treated with MI or MID were re-fed with medium containing insulin every other day as described in the Methods section. Results are representative of five independent experiments.

(B) Western analysis of adipsin protein levels in cells infected and treated as in (A) 8 days following induction to differentiate. Data shown is representative of 3 independent experiments.

(C) Glycerol-3-phosphate dehydrogenase assay results from extracts produced from cells infected and treated as in (A). Results are representative of 3 independent experiments performed in duplicate. Error bars represent the standard error of the mean.


A
pLXSN
GR505C
-C
+MI
+MID
B
pLXSN
GR505C
MI + + +
DEX - + +
adipsin
C
GPDH
(U/min/mg protein)
3500
3000
2500
2000
1500
1000
500
0
-C +MID -C +MID

domain or empty vector (pLXSN) do not differentiate or form lipid droplets (Fig. 7A). Similarly, treatment with MIX and insulin for the first 48 hrs of the differentiation protocol had no effect on cell morphology as evaluated by light microscopy. Treatment of the cells with the full differentiation cocktail containing MIX, insulin and dex resulted in a low level of differentiation in cells retrovirally infected with the empty vector. Expression of the GR ligand binding domain and treatment with the full cocktail increased the number of cells differentiated by approximately 3-fold. Adipsin was not detected by Western analysis 8 days after induction with MIX and insulin, though low levels were observed following treatment with the full differentiation cocktail (Fig. 7B). Consistent with the Oil red O results, retroviral expression of the GR ligand binding domain and treatment with MIX, insulin and dex resulted in an increase in adipsin detected (2.7 ± 0.23 fold) as compared to cells infected with empty virus, showing that these cultures differentiate more fully than those not expressing the GR ligand binding domain. Notably, the potentiating effect of the GR ligand binding domain depended on the treatment with steroid, as there was no observable difference in Oil red O staining detected in cultures treated with MIX and insulin alone.

Cell extracts were prepared in parallel experiments to analyze the activity of the glycerol 3-phosphate dehydrogenase enzyme (GPDH) (Fig. 7C). GPDH is a key enzyme in the metabolic pathway for the development of triacylglycerol in the adipocyte, and is considered a marker for the mature phenotype (Wise and Green, 1979). When mock-infected cells (pLXSN) were treated with MIX, insulin and dex, a 4-fold increase in GPDH activity was observed over cells treated with vehicle alone (-C) (Fig. 7C). In cells expressing the GR ligand binding domain, treatment with the full hormonal cocktail lead to an approximate 10-fold increase in GPDH activity when compared to vehicle alone, a full 2.5-fold increase over the mock infected cells treated with the differentiation cocktail.

The progesterone receptor ligand binding domain can substitute for the GR ligand binding domain during preadipocyte differentiation.

Although the GR ligand binding domain is able to potentiate differentiation of 3T3 L1 preadipocytes when treated with agonist, the sufficiency of the truncated receptor for the differentiation process is still undetermined. Since the 3T3 L1 express a full-length GR, it is impossible to determine the sufficiency of the LBD on this background.

The ligand binding domains of the steroid class of nuclear receptors share a highly conserved structure made up of 11 or 12 helices (Bledsoe et al., 2002). Alignment of the GR and progesterone receptor (PR) ligand binding domains reveals 70% homology (Fig. 8A) making these two receptors highly related. The progesterone receptor is not expressed in 3T3 L1 preadipocytes by Western analysis, though it can readily be detected in T47D extract used as a positive control (Fig. 8B). Expression of the ligand binding domain of the progesterone receptor in these cells therefore creates a system in which the sufficiency of the ligand binding domain can be evaluated, as the endogenous full length GR is not activated.

First, to evaluate the potential for the PR LBD to substitute for GR in the potentiation of C/EBPβ-dependent transcription, transient transfection assays in Cos7 cells, which lack expression of endogenous GR and PR were used. The PR ligand binding domain was able to potentiate C/EBPβ-mediated transcription from the C/EBPα promoter to a similar extent as the GR ligand binding domain when treated with agonist (12-fold) (Fig. 9A). In contrast, retinoic acid receptor α (RARα) was not able to potentiate C/EBPβ-mediated transcription suggesting that the transcriptional mechanism we are investigating was not a property of all nuclear hormone receptors. These results suggested that the PR ligand binding domain may be able to substitute for GR in the adipogenesis system, as C/EBPα transcription was equally influenced by both receptor ligand binding domains in transcription assays.

Figure 8. The progesterone ligand binding domain can enhance transcription from the C/EBPα promoter.

(A) Amino acid sequence alignment of the rat glucocorticoid receptor hinge and ligand binding domain (GR amino acids 505-795) and the human progesterone receptor hinge and ligand binding domain (PR amino acids 632-933). The receptor ligand binding domains share 70% homology and 50% identity. Similarities between the two sequences are shown in the space between the individual receptor sequences. A letter designation indicates identical residues and conserved residues are indicated by a plus sign.

(B) Whole cell extract (50 μg) from 3T3 L1 and T47D cells was used for Western analysis of endogenous progesterone receptor expression.

A

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GR 505 MNLEARKTKK--KIKGIQQATAGVSQDTSENPNK --------TIVPAALPQLTPT
       M L  RK KK  K++ ++   A         PN+        T  P    QL P
PR 632 MVLGGRKFKKFNKVRVVRALDAVALPQPVGVPNESQALSQRFTFSPGQDIQLIPP

       LVSLLEVIEPEVLYAGYDSSVPDSAWRIMTTLNMLGGRQVIAAVKWAKAILGLRN
       L++LL  IEP+V+YAG+D++ PD++  ++T+LN LG RQ+++ VKW+K++ G RN
       LINLLMSIEPDVIYAGHDNTKPDTSSSLLTSLNQLGERQLLSVVKWSKSLPGFRN

       LHLDDQMTLLQYSWMFLMAFALGWRSYRQSSGNLLCFAPDLIINEQRMSLPCMYD
       LH+DDQ+TL+QYSWM LM F LGWRSY+  SG +L FAPDLI+NEQRM     Y
       LHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFAPDLILNEQRMKESSFYS

       QCKHMLFVSSELQRLQVSYEEYLCMKTLLLLSSVPKEGLKSQELFDEIRMTYIKE
        C  M  +  E  +LQVS EE+LCMK LLLL+++P EGL+SQ  F+E+R +YI+E
       LCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQTQFEEMRSSYIRE

       LGKAIVKREGNSSQNWQRFYQLTKLLDSMHEVVENLLTYCFQTFL -DKTMSIEFP
       L KAI  R+     + QRFYQLTKLLD++H++V+ L  YC  TF+  + +S+EFP
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B

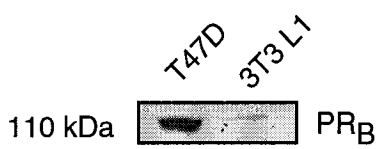
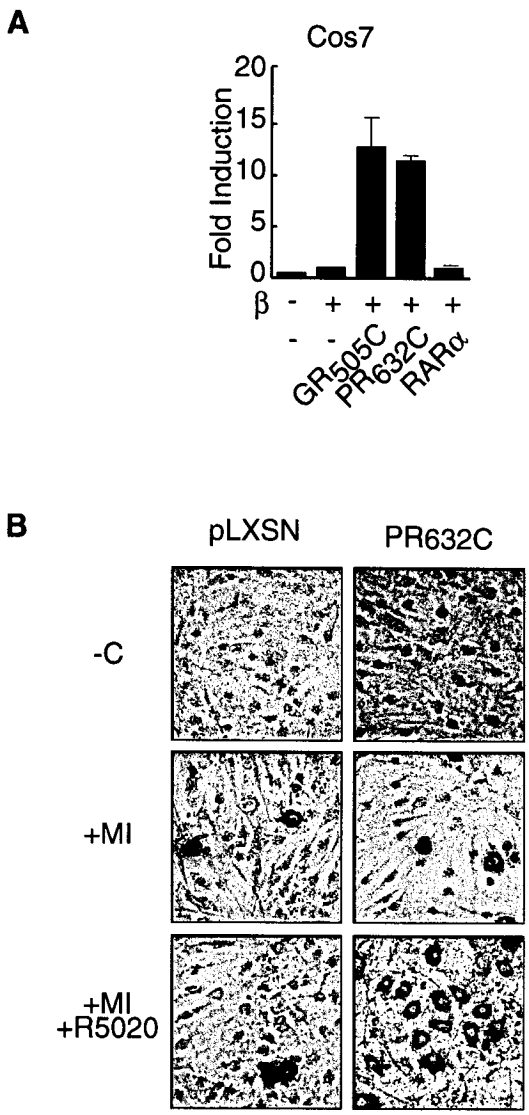


Figure 9. The progesterone receptor LBD enhances adipogenesis when progestins are substituted for dex in the differentiation cocktail.

(A) The PR LBD can potentiate C/EBPβ-mediated transcription from the C/EBPα promoter. Cos7 cells were transfected with βgal, pCX14/12, pMSVC/EBPβ and 100ng of GR_{505C}, the human progesterone receptor LBD (PR_{632C}), or full length human RARα as indicated. Cells were treated for 24 hrs with vehicle, dex, R5020 or all trans retinoic acid as required. Data is represented as fold induction over C/EBPβ transfection alone.

(B) Oil Red O staining of 3T3 L1 cells infected with control virus (pLXSN) or with virus to express the PR LBD (PR_{632C}) 8 days after treatment with MIX and Insulin (+MI), MIX, Insulin and R5020 (+MI+R5020) or in the absence of cocktail (-C) for the first 48hrs of the differentiation protocol. Cells treated with MI or MI+R5020 were re-fed with medium containing insulin every other day as described in the methods section. Results are representative of five independent experiments.


A
Cos7
Fold Induction
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β - + + + +
- - GR505C PR632C RARα
B
pLXSN
PR632C
-C
+MI
+MI
+R5020

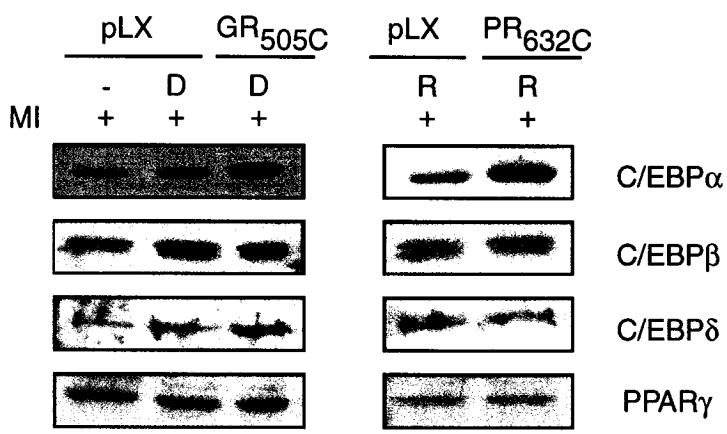
Expression of the PR ligand binding domain in 3T3 L1 preadipocytes resulted in a significant increase in the number of cells staining positive for neutral lipid by Oil red O following agonist treatment when compared to cells treated with vehicle (Fig. 9B). This effect was dependent on treatment with the agonist R5020, as there was no increase in differentiation observed in cells treated with MIX and insulin alone. Infection of the preadipocytes with the control virus lacking an insert (pLXSN) did not lead to an increase in differentiation even upon treatment with MIX, insulin and R5020. Thus, the effect of progestins was dependent on the presence of the PR ligand binding domain.

Retroviral expression of GR and PR ligand binding domains correlates with increased C/EBPα protein levels.

3T3 L1 cells retrovirally expressing either the GR or PR ligand binding domains and induced to differentiate with MIX, insulin and the appropriate agonist (dex or R5020) for 24 hrs expressed higher levels of C/EBPα protein than cells not expressing a receptor LBD (Fig. 10). Cells expressing the GR LBD expressed 2.4±0.02 fold greater C/EBPα levels than cells infected with empty virus and induced to differentiate with the dex-containing treatment. Expression of the PR LBD led to an increase of C/EBPα expression of 2.20±0.04 fold. No differences in C/EBPβ or C/EBPδ levels were observed in these cultures. The PPARγ protein levels were equally unaffected by expression of the receptor ligand binding domains, as predicted by the results of the transient transcription experiments performed with this promoter.

Figure 10. Expression of the GR or PR ligand binding domain increases C/EBPα expression in differentiating 3T3 L1 preadipocytes.

3T3 L1 cells infected and treated as in figure 7A and figure 9B were harvested 24 hrs following induction to differentiate with MIX and insulin (MI) in the presence or absence of dex (D) or R5020 (R) as required. Whole cell extracts (50 μg) were separated by SDS-PAGE and expression levels of C/EBPβ, C/EBPδ, C/EBPα and PPARγ were evaluated using Western analysis and analyzed by phosphorimager. Blots are representative of a minimum of 3 independent experiments.


pLX
GR505C
pLX
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C/EBPα
C/EBPβ
C/EBPδ
PPARγ

Transcription by C/EBPβ is repressed by Histone Deacetylase 1

Since the GR ligand binding domain is not able to contact DNA directly, and previous work has indicated that it does not interact with C/EBPβ (Boruk et al., 1998), it was hypothesized that GR was acting to influence coactivator or corepressor molecules associated with DNA-bound C/EBPβ. C/EBPβ has been previously shown to interact, and to be coactivated by p300 (Mink et al., 1997) and members of the Swi/Snf family (Kowenz-Leutz and Leutz, 1999), via interaction with the N-terminus of the C/EBPβ protein. In transient transcription experiments, treatment with a histone deacetylase inhibitor (trichostatin A) lead to a robust increase (53-fold) in transcription by C/EBPβ from the C/EBPα promoter, and no additional effect was observed when cells were treated with both dex and trichostatin A (Fig. 11A). The increase in transcription indicated that histone deacetylases play a role in inhibiting transcription by C/EBPβ. Because treatment with dex has no additional effect, it suggests that GR and the histone deacetylase inhibitor are influencing the same pathway to release C/EBPβ from a repressive force.

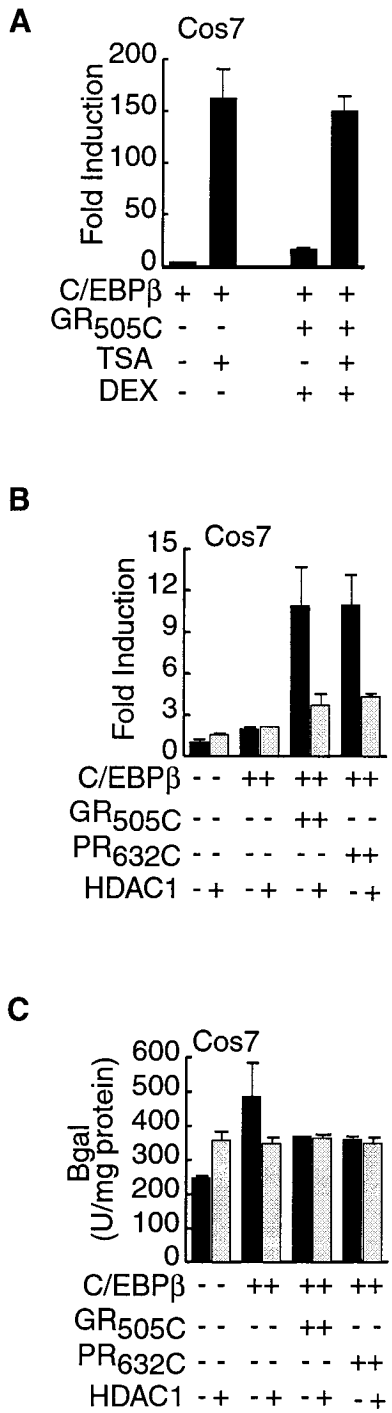
Published microarray data indicated that HDAC1 is downregulated at the RNA level 48 hrs following induction of differentiation with MIX, insulin and dex (Soukas et al., 2001). This suggested HDAC1 as a candidate deacetylase for repressing C/EBPβ during the early phase of differentiation, whose action is titrated from the system at later phases of differentiation. To evaluate whether HDAC1 could repress C/EBPβ-dependent transcription from the C/EBPα promoter, the effect of ectopic expression of HDAC1 in transient transcription assays was evaluated. Ectopic HDAC1 was unable to repress transcription by C/EBPβ in this system (Fig. 11B). However, HDAC1 strongly inhibited the potentiation of transcription produced by the agonist treated GR and PR ligand binding domains by approximately 3-fold (Fig. 11B). This result suggested that the agonist-bound receptor LBDs act to relieve HDAC1 repression of

Figure 11. HDAC1 blocks the effect of dex on C/EBPβ-mediated transcription.

(A) Treatment with a deacetylase inhibitor TSA greatly increases transcription by C/EBPβ from the C/EBPα promoter. Cos7 cells were transfected with βgal, pCX14/12, pMSVC/EBPβ and GR_{505C} as indicated. Cells were treated with dex and trichostatin A (TSA) for 24hrs prior to harvesting for luciferase assay.

(B) Cos 7 cells were transfected with βgal, C/EBPα reporter construct, C/EBPβ, GR_{505C}, PR_{632C} and 300ng HDAC1 (pCDNA3.1-HDAC1) as indicated. Cells were treated with vehicle, dex or R5020 for 24 hrs as indicated. Data are represented as fold induction over basal promoter activity.

(C) βgal activities corrected for total protein from extracts in (B) indicating that HDAC1 has no effect on the Rous sarcoma virus promoter driving βgal expression. Results are shown as βgal activity corrected for total protein.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TSA - + - +
DEX - - + +
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Cos7
Fold Induction
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C/EBPβ - - ++ ++ ++
GR505C - - - - ++ - -
PR632C - - - - - - ++
HDAC1 - + - + - + - +
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C/EBPβ - - ++ ++ ++
GR505C - - - - ++ - -
PR632C - - - - - - ++
HDAC1 - + - + - + - +

C/EBPβ activity, as addition of ectopic HDAC1 reversed the positive effects on transcription. In the absence of the receptor LBDs, C/EBPβ is in a repressed state that is not further repressed by additional HDAC1 protein. The effect of HDAC1 on transcription was specific for C/EBPα transcription as no effect of ectopic HDAC1 was observed on βgal transcription, driven by the RSV promoter (Fig. 11C).

Overexpression of HDAC1 blocks preadipocyte differentiation.

Retroviral expression of HDAC1 in 3T3 L1 preadipocytes inhibited differentiation following induction by MIX, insulin and dex (Fig. 12A). Cells infected with the empty vector (pLXSN) differentiated minimally (approximately 5%) following induction to differentiate with MIX and insulin. Treatment of these cultures with the full differentiation cocktail including dexamethasone resulted in robust differentiation of the cultures, as evidenced by the Oil red O staining. Retroviral expression of murine HDAC1 completely blocked differentiation in these cells, even after treatment with the full differentiation cocktail. Western analysis of adipsin from these cells 8 days following induction to differentiate in the presence of HDAC1 demonstrated a marked decrease in this marker's expression (4.65±0.07 fold decrease) (Fig. 12B). This block in differentiation was not due to decreased induction of C/EBPβ, as C/EBPβ protein levels were equal in mock-infected cells and in cells expressing HDAC1 (Fig. 12C). Therefore, the defect in the differentiation process caused by HDAC1 occurs downstream of C/EBPβ upregulation by MIX treatment. Indeed, expression of C/EBPα was greatly reduced (5.18±0.31 fold decrease) 24 hours following induction to differentiate in cells retrovirally expressing HDAC1 (Fig. 12D). C/EBPδ levels were unaffected by expression of HDAC1 suggesting that the defect in adipogenesis follows induction of the early markers C/EBPβ and C/EBPδ.

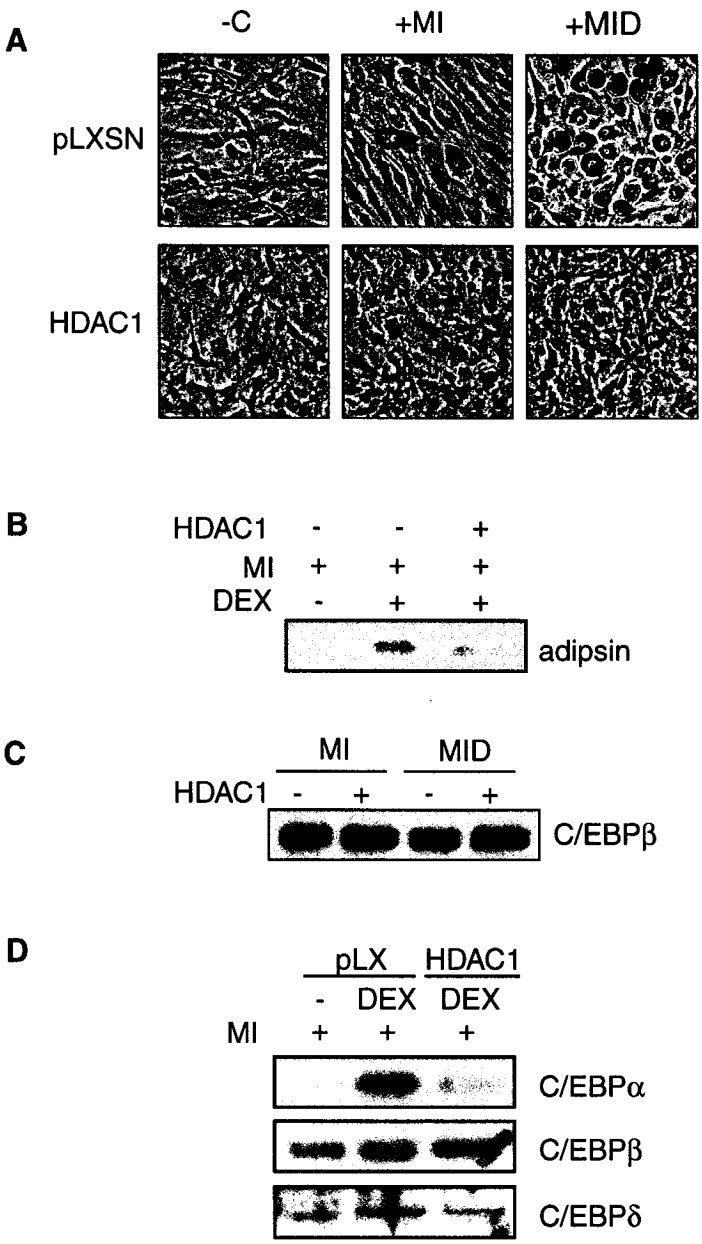
Figure 12. HDAC1 blocks preadipocyte differentiation and expression of C/EBPα.

(A) Oil red O staining of 3T3 L1 cells infected with control virus (pLXSN) or to express HDAC1 8 days after induction to differentiate with MIX and insulin (+MI) or MIX, insulin and dex (+MID). Results are representative of 3 independent experiments.

(B) Western analysis of infected 3T3 L1 cells from (A) 8 days following induction to differentiate with MIX and insulin and dex as indicated. 100μg of extract was used for analysis and blots were probed with an anti-adipsin antibody.

(C) Western analysis of C/EBPβ protein levels in cells infected and induced to differentiate for 24 hours as in (A).

(D) Western analysis of C/EBPα, β and δ protein levels in cells infected and induced to differentiate for 24 hours as in (A).


A
-C
+MI
+MID
pLXSN
HDAC1
B
HDAC1 - - +
MI + + +
DEX - + +
adipsin
C
MI
MID
HDAC1 - + - +
C/EBPβ
D
pLX
HDAC1
- DEX DEX
MI + + +
C/EBPα
C/EBPβ
C/EBPδ

In a further experiment, histone deacetylase inhibitors trichostatin A (TSA) and valproic acid (VPA) replaced dex in the differentiation cocktail. Valproic acid is a pharmacologic agent used to treat mood and seizure disorders that was recently identified as an HDAC inhibitor (Gottlicher et al., 2001; Phiel et al., 2001). Interestingly, one of the common side effects of treatment of patients with VPA is obesity (Biton et al., 2001; Dinesen et al., 1984; Wolden-Hanson et al., 1998). Cells treated with MIX, insulin and TSA, or MIX, insulin and VPA differentiated to the same extent as cells treated with the standard differentiation cocktail including MIX, insulin and dex (Fig. 13A). Treatment with dex in addition to the histone deacetylase inhibitors had no further effect on differentiation, again suggesting a common pathway of action for the inhibitors and dexamethasone. Adipsin levels were significantly increased following treatment with TSA or VPA (42.5±3.39 fold and 12.7±0.11 fold respectively) (Fig. 13B) 8 days after induction to differentiate as were C/EBPα levels 24 hrs after induction (13.96±0.24 and 8.98±0.23 respectively)(Fig. 13C). C/EBPβ and δ levels were unaffected by the histone deacetylase inhibitors.

C/EBPβ associates with an HDAC-containing subcomplex of the Sin3 corepressor complex

HDAC1 has been characterized to function as part of several multiprotein corepressor complexes (Ayer, 1999; You et al., 2001). To evaluate if C/EBPβ interacted with HDAC1 *in vivo*, coimmunoprecipitation experiments were performed from Cos7 cells transiently expressing C/EBPβ and from differentiating 3T3 L1 preadipocytes. In Cos7 cells transiently expressing C/EBPβ, immunoprecipitation of C/EBPβ revealed an interaction with endogenous HDAC1 (Fig. 14A) as well as with p300 reported previously. Similar results were obtained in 3T3 L1 preadipocytes when extracts were prepared from cells induced to differentiate for 24 hrs with MIX and insulin (Fig. 14B).

Figure 13. Deacetylase inhibitors replace dexamethasone during preadipocyte differentiation.

(A) Oil red O staining of 3T3 L1 8 days after induction to differentiate with MIX and Insulin (+MI), Mix, Insulin and Trichostatin A (+MI+TSA) or Mix. Insulin and valproic acid (+MI+VPA) in the presence or absence of dex. Results are representative of 3 independent experiments.

(B) Western analysis of infected 3T3 L1 cells from (A) 8 days following induction to differentiate with MIX and insulin and dex as indicated. 100μg of extract was used for analysis and blots were probed with an anti-adipsin antibody. Results are representative of 3 independent experiments.

(C) Western analysis of C/EBPα, C/EBPβ and PPARγ protein levels in cells infected and induced to differentiate for 24 hours as in (A). Results are representative of 3 independent experiments.

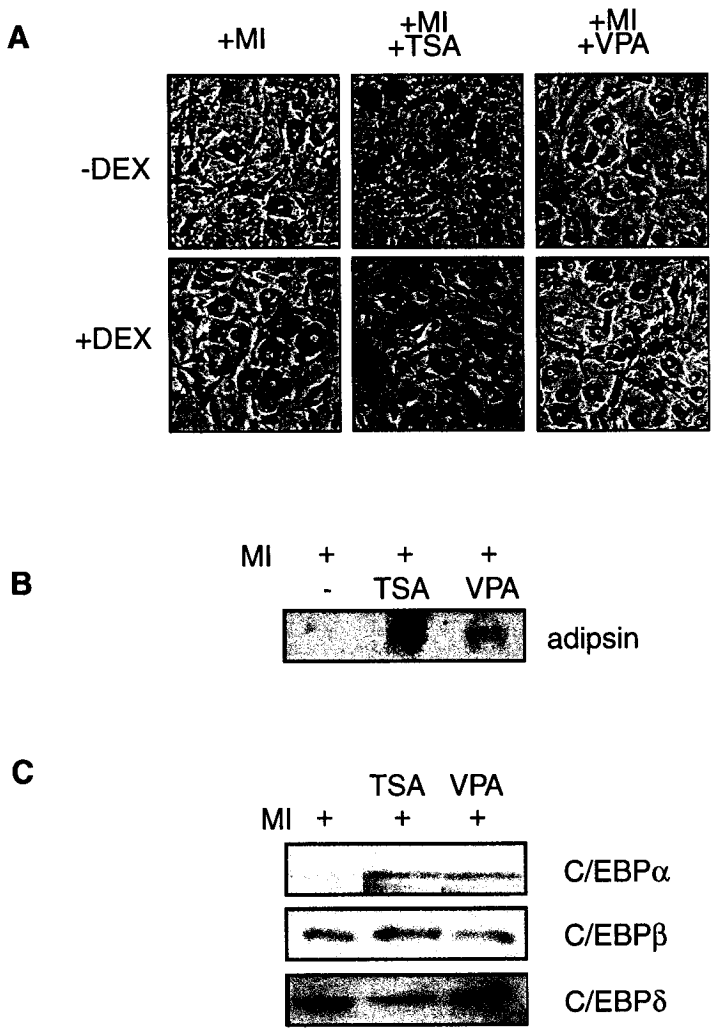
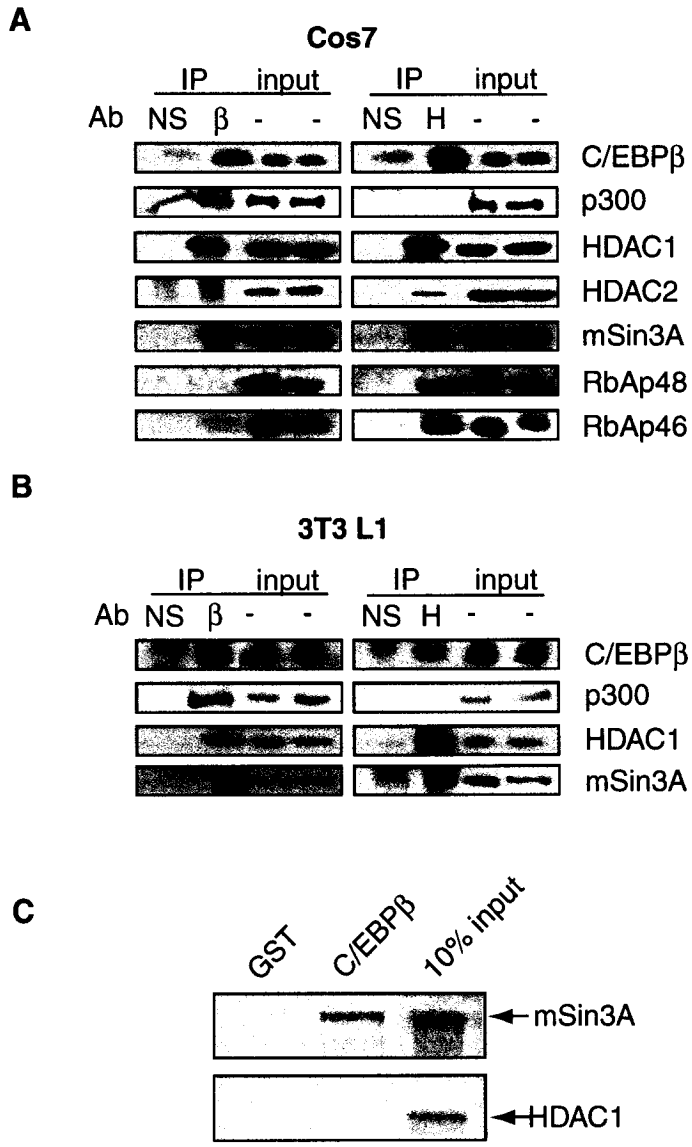

A
+MI
+MI
+TSA
+MI
+VPA
-DEX
+DEX
B
MI + + +
- TSA VPA
adipsin
C
TSA VPA
MI + + +
C/EBPα
C/EBPβ
C/EBPδ

Figure 14. C/EBPβ interacts with a novel subcomplex of the Sin3 corepressor complex.

(A) Coimmunoprecipitation of the subcomplex with C/EBPβ. Cos7 cells were transfected with C/EBPβ and 48 hrs post-transfection were harvested. One milligram of whole cell extract was immunoprecipitated with anti-C/EBPβ (β), anti-HDAC1 (H) or type-matched non-specific (NS) antibody. Western blots were probed with the indicated antibodies. Inputs represent 10% of the extract used for immunoprecipitation.

(B) Coimmunoprecipitation experiments as in (A) from extracts prepared from 3T3 L1 cells treated with MIX and Insulin for 24 hrs following confluency.

(C) GST pulldown assay of in vitro translated ^{35}S-labelled mSin3A and HDAC1 with GST alone and GST-C/EBPβ. Input represents 10% of the radiolabelled protein used in the binding reaction.


A
Cos7
IP input IP input
Ab NS β - - NS H - -
C/EBPβ
p300
HDAC1
HDAC2
mSin3A
RbAp48
RbAp46
B
3T3 L1
IP input IP input
Ab NS β - - NS H - -
C/EBPβ
p300
HDAC1
mSin3A
C
GST
C/EBPβ
10% input
mSin3A
HDAC1

To evaluate the context of the HDAC1 interaction, the interaction of other corepressor molecules previously identified to be part of HDAC1-containing complexes were examined by coimmunoprecipitation. HDAC2, which is found in all of the previously characterized HDAC1-containing complexes (NuRD, Sin3 and CoREST) was not observed in C/EBPβ immunoprecipitates from Cos7 cells, though it readily coprecipitated with HDAC1 from these same cells (Fig. 14A). Therefore, although HDAC1 and HDAC2 containing complexes do exist in Cos7 cells, HDAC2 is not part of a potential C/EBPβ-interacting complex. This result effectively eliminated the known Sin3, NuRD and CoREST complexes as interacting with C/EBPβ. In fact, the chromatin binding proteins RbAp46 and RbAp48, found in both the Sin3 and NuRD complexes did not interact with immunoprecipitated C/EBPβ, though both were present in HDAC1 precipitates. However, mSin3A was observed to interact with C/EBPβ in both Cos7 cells and 3T3 L1 preadipocytes (Fig. 14A and B). Together, these results supported the interaction of C/EBPβ with a subcomplex of the known Sin3 complex which lacks HDAC2 and the chromatin binding proteins RbAp46 and RbAp48.

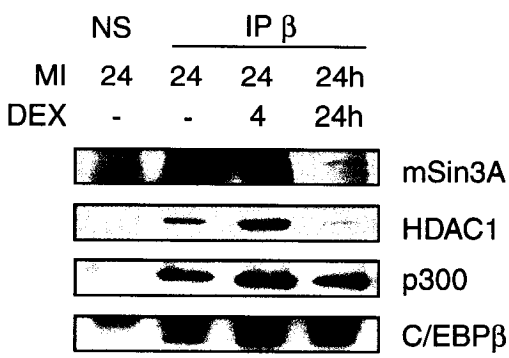
GST pulldown assays indicated that *in vitro* translated ^{35}S-labelled HDAC1 was unable to interact with GST-C/EBPβ or GST alone (Fig. 14C). However, ^{35}S-labelled mSin3A interacted strongly with GST-C/EBPβ but did not interact with GST alone, a result that suggests that mSin3A may serve as an anchor point for HDAC1 association.

HDAC1 is degraded by the 26S proteasome following dex treatment.

Treatment of 3T3 L1 cells with dex for 24 hrs resulted in a loss of the interaction observed between C/EBPβ and mSin3A and HDAC1 (Fig. 15). Coimmunoprecipitated C/EBPβ interacted with p300 in the absence of dex treatment and equally after a 4 hr and 24 hr steroid

Figure 15. Steroid treatment decreases HDAC1 association with C/EBPβ.

Co-immunoprecipitation of HDAC1 and mSin3A with C/EBPβ is decreased with DEX treatment. 3T3 L1 cells were treated with MIX and Insulin (MI) and vehicle or DEX for 24 hrs or for only the last 4 hrs of the 24 hr MI treatment.


NS
IP β
MI 24 24 24 24h
DEX - - 4 24h
mSin3A
HDAC1
p300
C/EBPβ

treatment. Both mSin3A and HDAC1 also interacted with C/EBPβ in the absence of dex (Fig. 14A and B), and following a 4 hr dex treatment (Fig. 15). Twenty-four hour dex treatment resulted in a strong reduction in the amount of mSin3A and HDAC1 coprecipitated though p300 coprecipitates were unaffected.

In two-day post-confluent 3T3 L1 cells, treatment with MIX, insulin and dex caused a 50% reduction (51.6 ± 5.7%) in HDAC1 protein levels by Western analysis, potentially explaining the loss of coprecipitation with C/EBPβ observed following prolonged steroid treatment (24 hrs) (Fig. 16A). This decrease in protein level was prevented by addition of the proteasome inhibitor MG132 (91.3 ± 6.7% of MG132 treatment alone) (Fig. 16A). Higher molecular weight species that cross-react with the HDAC1 antibody can be observed following MG132 treatment, and may represent ubiquitylated forms of HDAC1. Interestingly, mSin3A protein levels were unaffected by dex treatment and were not noticeably stabilized by treatment with MG132. The glucocorticoid receptor itself, as previously reported (Wallace and Cidlowski, 2001) was almost completely degraded following steroid treatment. The degradation was only minimally rescued by treatment with proteasome inhibitor.

Retroviral expression of the PR ligand binding domain in the 3T3 L1 preadipocytes and substitution of R5020 for dex in the treatment cocktail resulted in a similar decrease in HDAC1 protein levels (47.9±2.7%), whose loss was once again prevented by proteasome inhibitor treatment (Fig. 16B). Higher molecular weight forms of HDAC1 were once again observed under these conditions. The degradation of HDAC1 was also observed in Cos7 cells transiently expressing the GR ligand binding domain (52.6±0.1%) (Fig 16C, top) and was even more pronounced when ectopic HA-tagged HDAC1 was coexpressed (67.0±1.1%) (Fig. 16C, bottom). This decrease in HDAC1 protein levels was not due to regulation at the transcription level, as HDAC1 mRNA levels were actually induced over the12 hours following treatment with MIX, insulin and dex, and remained constant thereafter up to 36 hours (Fig. 16D) by Northern analysis.

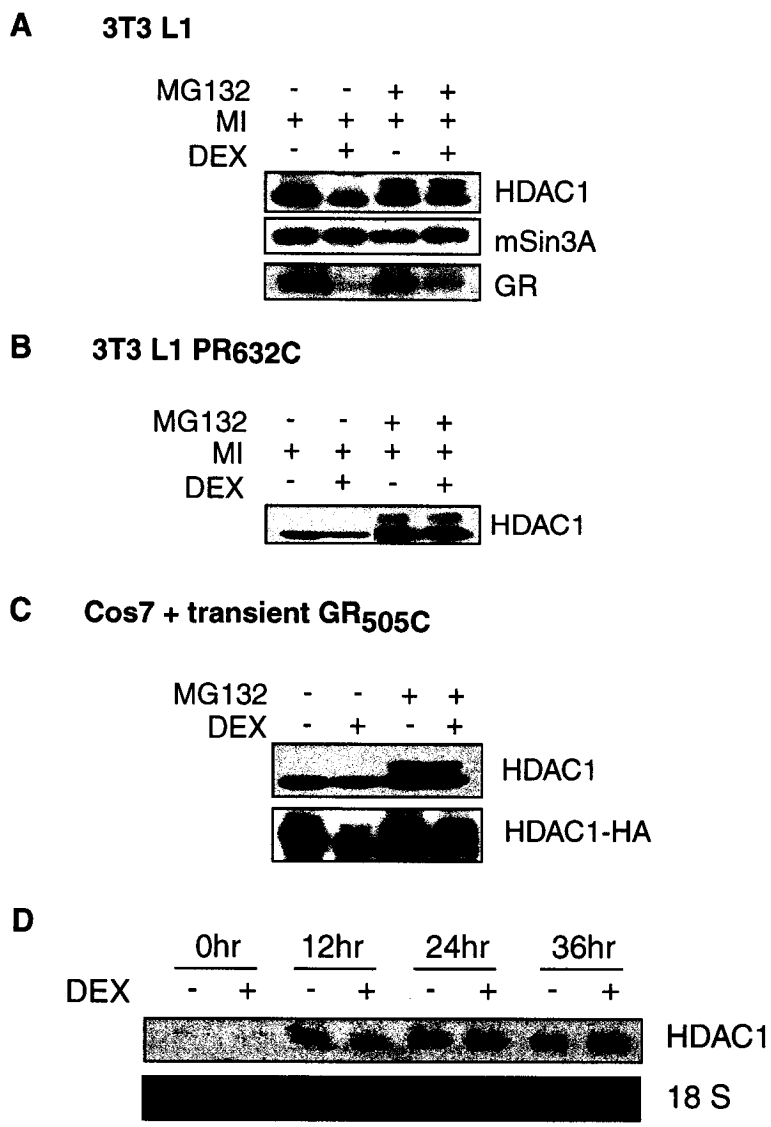
Figure 16. Steroid treatment destabilizes HDAC1 levels.

(A) Western analysis of protein levels following treatment with DEX and MG132. 3T3 L1 cells were treated with MIX, Insulin (+MI) or MIX, Insulin and DEX (+MID) in the presence or absence of the proteasome inhibitor MG132.

(B) Western analysis of HDAC1 protein levels in 3T3 L1 cells infected to express the progesterone receptor ligand binding domain (PR_{632C}). Cells were treated with MIX and insulin or MIX, insulin and R5020 (+MIR) in the presence or absence of MG132.

(C) Western analysis of HDAC1 protein levels in Cos7 cells transiently expressing the GR ligand binding domain (GR_{505C}). The upper panel represents changes in endogenous HDAC1 levels upon DEX and MG132 treatment. The bottom panel represents changes in ectopic HA-tagged HDAC1 levels.

(D) Northern analysis of HDAC1 mRNA of 3T3 L1 cells following the induction to differentiate with MIX and insulin in the presence (+) or absence (-) of dex over 36 hours..


A
3T3 L1
MG132 - - + +
MI + + + +
DEX - + - +
HDAC1
mSin3A
GR
B
3T3 L1 PR632C
MG132 - - + +
MI + + + +
DEX - + - +
HDAC1
C
Cos7 + transient GR505C
MG132 - - + +
DEX - + - +
HDAC1
HDAC1-HA
D
0hr 12hr 24hr 36hr
DEX - + - + - + - +
HDAC1
18 S

Published microarray data indicates that the HDAC1 mRNA levels begin to fall rapidly 48 hrs after treatment with the differentiation cocktail (Soukas et al., 2001).

A C/EBPβ-interacting mSin3A-HDAC1 complex is specifically lost following dex treatment.

Immunoprecipitation of RbAp46, a component of both the NuRD and mSin3A corepressor complexes, from 3T3 L1 cells induced to differentiate for 24 hours revealed a constant association of HDAC1 with the corepressor even following dex treatment (Fig. 17A). These results suggest that despite a reduction of cellular HDAC1 levels by approximately half certain HDAC1 containing complexes were unaffected in their HDAC1 content following steroid treatment. However, the 50% reduction in HDAC1 protein levels 24 hrs following dex treatment results in a complete loss of interaction of HDAC1 with C/EBPβ (Fig. 15).

Size exclusion chromatographic separation of HDAC1-containing complexes from 3T3 L1 cells treated for 24 hrs with MIX and insulin revealed the presence of 6 HDAC1-containing peaks (peaks I-VI) (Fig. 17B). These peaks differ in their HDAC1 content as well as the comigration of other corepressor molecules such as mSin3A and RbAp48 (Fig. 17C). Addition of dex to the differentiation cocktail resulted in a mobility shift of peak IV (now referred to as peak IVa) and a complete loss of peak VI (Fig. 17B). Peak VI contained the majority of the cellular C/EBPβ (49%) and a significant proportion of the HDAC1 (36%) (Fig. 17C). This peak also contained mSin3A and was free of RbAp48, consistent with the C/EBPβ-interacting complex identified by coimmunoprecipitation (Fig. 14A). The HDAC1 content of peak V was also reduced by approximately half, though its elution from the column was unaffected. These results demonstrate that the loss of approximately 50% of cellular HDAC1 observed by Western analysis following dex treatment corresponds to a complete loss of the HDAC1 found in a C/EBPβ-interacting complex and a 50% reduction of HDAC1 content of a second uncharacterized peak.

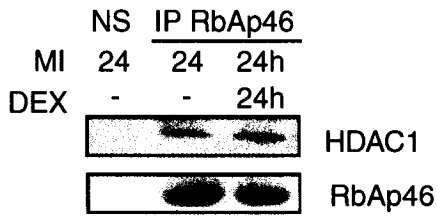
Figure 17. C/EBPβ preferentially interacts with a subpopulation of HDAC1 that is degraded following dex treatment.

(A) Coimmunoprecipitation of HDAC1 with RbAp46 from 3T3 L1 cells induced to differentiate with MIX and Insulin and dex as indicated.

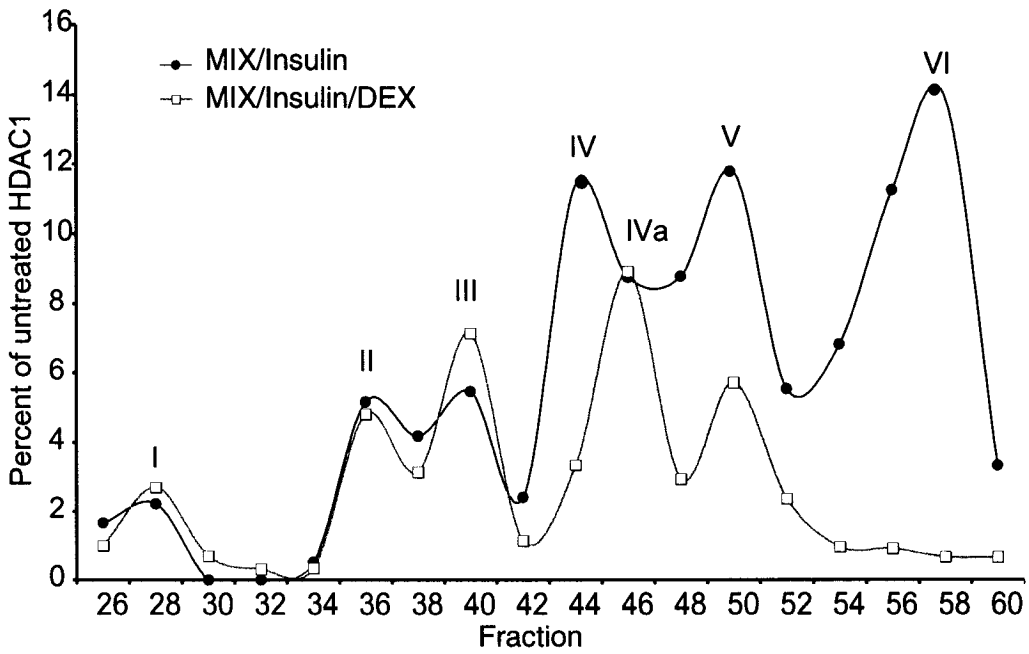
(B) FPLC separation of HDAC1-containing complexes by size exclusion chromatography. Six different complexes were observed in 3T3 L1 extracts prepared following a 24 hr treatment with MIX and insulin (dark circles) or MIX,insulin and dex (open squares). Western analysis of HDAC1 contents in every second fraction was performed and analyzed by phosphorimager. Data is representative of the percent of HDAC1 in an untreated sample as determined by Western analysis and quantification by phosphorimager of total HDAC1 in cell extracts before separation. Upon dex treatment, a small shift in the migration of peak IV was observed, and was named IVa.

(C) Relative distribution of HDAC1, mSin3A, RbAp48 and C/EBPβ from cells treated with MIX and insulin for 24 hr in the different peaks as determined by phosphorimager analysis.

A



B



C

Fraction	I	II	III	IV	V	VI
%HDAC1	3	6	10	20	24	36
%mSin3A	-	6	-	24	-	34
%RbAp48	-	5	21	10	17	-
%C/EBPβ	17	-	12	12	10	49

The glucocorticoid receptor interacts in a ligand dependent manner with mSin3A and HDAC1.

The mechanism by which the glucocorticoid receptor targets the HDAC1 for degradation is unknown, but by virtue of its own ligand dependent degradation we suspected that it may ferry C/EBPβ-associated HDAC1 to the proteasome. Coimmunoprecipitations from 3T3 L1 cells revealed that GR could interact with both mSin3A and HDAC1 in a ligand dependent manner (Fig 18). Following a 24 hr treatment with MIX and insulin, the receptor is seen to interact minimally with mSin3A. This is believed to be due to the use of whole cell extracts in these experiments, as unliganded GR is confined to the cytoplasm, and mSin3A is a nuclear protein. Despite the use of molybdate to prevent release of the receptor from the heat shock proteins it is possible that some release of the receptor occurred during manipulation of the whole cell extracts. When dex was added to the differentiation cocktail for the final 4 hrs, interactions of p300, HDAC1 and mSin3A with the receptor were observed (Fig. 18A). Binding of mSin3A and HDAC1 to GR occurs through the ligand binding domain. Immunoprecipitation of the GR ligand binding domain (GR_{505C}) transiently expressed in Cos7 cells coprecipitated HDAC1 (Fig. 18B).

Treatment with dexamethasone results in loss of HDAC1 from the C/EBPα promoter and histone acetylation.

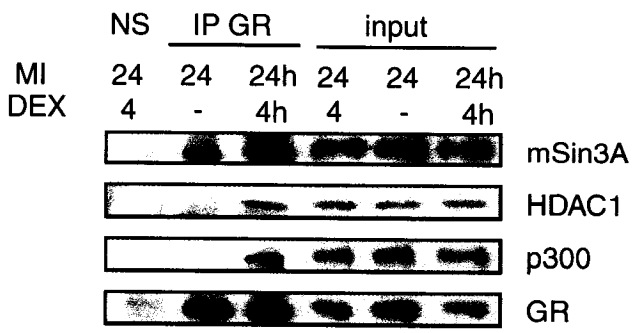
To assess how loss of the C/EBPβ-interacting HDAC1 complex impacted transcription, chromatin immunoprecipitation of factors associated with the C/EBP motif of the C/EBPα promoter were performed (Fig. 19A). Untreated post-confluent 3T3 L1 cells (-C) do not upregulate C/EBPβ protein levels, and thus as expected, C/EBPβ was not detected on the C/EBPα promoter. Other C/EBPβ associated factors such as p300 were also not detected at the promoter under these conditions. Treatment of two-day post-confluent 3T3 L1 cells with MIX

Figure 18. The glucocorticoid receptor interacts with HDAC1 and mSin3A in vivo.

(A) 3T3 L1 cells were treated with MIX/insulin in the presence or absence of DEX for the last 4 hrs of the 24 hr treatment. GR was immunoprecipitated and blots were probed with the indicated antibodies. Input represents 10% of the extract used for immunoprecipitation.

(B) Coimmunoprecipitation of HDAC1 with transiently expressed GR ligand binding domain (GR_{505C}) in Cos7 cells. Transfected cells were treated with DEX for 4 hrs as indicated.

A **3T3 L1**



B **Cos7 + GR_{505C}**

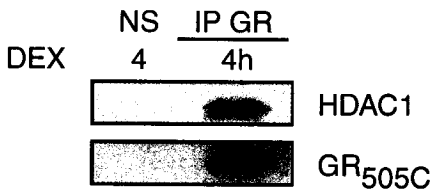
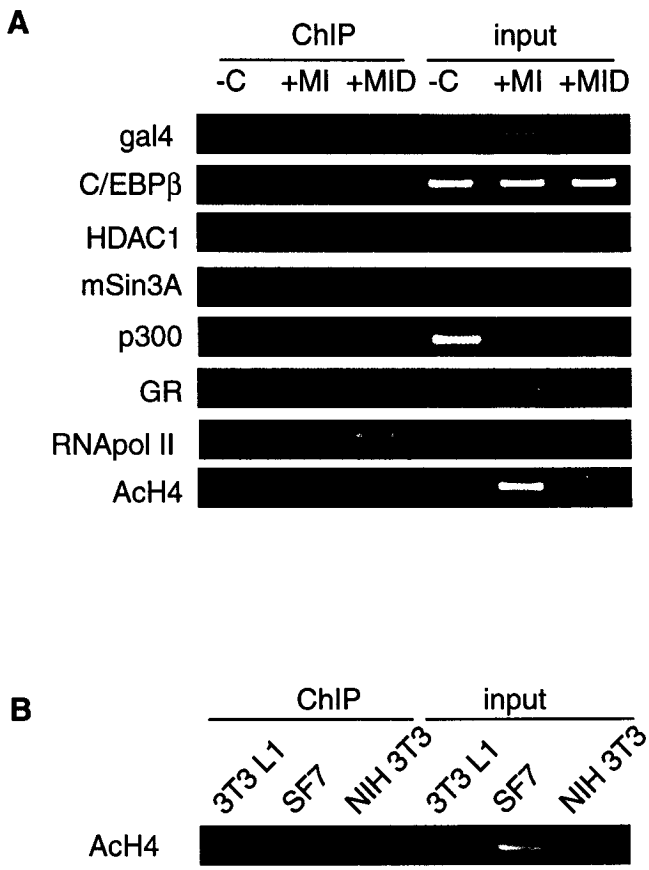


Figure 19. HDAC1 and mSin3A are lost from the C/EBPα promoter following DEX treatment.

(A) Chromatin immunoprecipitation (ChIP) experiments from 3T3 L1 cells treated with vehicle (-C), MIX/insulin (+MI), or MIX/insulin/DEX (+MID) for 24 hrs. Immunoprecipitations were performed with the indicated antibodies. Gal4 was used as a non-specific antibody, RNA pol II is an antibody raised against RNA polymerase II and the AcH4 antibody is raised against acetylated histone H4. Inputs represent 5% of the material used for immunoprecipitation. Immunoprecipitates and input samples were amplified by polymerase chain reaction using oligonucleotides for positions –334 to –118 spanning the C/EBP motif and positions –108 to +17 spanning the TATA box for analysis of RNA pol II association. Results are representative of a minimum of 3 independent experiments.

(B) ChIP experiment examining the histone H4 acetylation status in untreated 3T3 L1 cells and the mouse fibroblast cell lines SF7 and NIH 3T3. All cell lines were analyzed at confluency. Results are representative of 3 independent experiments.


A
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+MI
+MID
-C
+MI
+MID
gal4
C/EBPβ
HDAC1
mSin3A
p300
GR
RNApol II
AcH4
B
ChIP
input
3T3 L1
SF7
NIH 3T3
3T3 L1
SF7
NIH 3T3
AcH4

and insulin resulted in association of C/EBPβ with the promoter element. HDAC1, mSin3A and p300 were similarly recruited to the promoter (Fig. 19A). The recruitment of HDAC1 correlated with no detectable acetylation of histone H4 and no recruitment of RNA polymerase II to the TATA box. These results suggest that the p300 found at the promoter was insufficient to overcome the inhibitory effects of HDAC1. Addition of dex to the treatment cocktail resulted in recruitment of C/EBPβ to the promoter but HDAC1 and mSin3A were not detected, consistent with the coimmunoprecipitation results. The association of p300 with the promoter was unchanged in the presence of steroid. Under these conditions, acetylation of histone H4 and recruitment of RNA polymerase II was observed, indicating a transcriptionally active promoter and reflecting the shift in equilibrium favouring histone acetyltransferases. GR was not detected at the promoter under any treatment conditions, again suggesting that its role is non-transcriptional in nature.

Interestingly, histone H4 was observed to be acetylated in untreated 3T3 L1 preadipocytes, a modification that is reversed upon recruitment of C/EBPβ and the associated HDAC1. Comparison of histone H4 acetylation in different murine fibroblastic cell lines (SF7 and NIH 3T3) indicated that this acetylation was a property of 3T3 L1 preadipocytes and may be representative of their commitment to the adipocyte lineage (Fig. 19B). NIH 3T3 cells are murine fibroblasts derived from day 17 embryos, which, upon forced expression of C/EBPβ and treatment with the full differentiation cocktail of MIX, insulin and dex can differentiate into mature adipocytes.

Treatment with the proteasome inhibitor MG132 prevents loss of HDAC1 from the C/EBPα promoter and represses transcription.

Cotreatment of 3T3 L1 preadipocytes with dex and the proteasome inhibitor MG132 prevented the degradation of HDAC1 by the proteasome (Fig. 16A). Chromatin

immunoprecipitations from 3T3 L1 cells induced to differentiate in the presence of MG132 revealed an association of HDAC1 with the C/EBPα promoter that was dependent on MIX and insulin and which persisted following dex treatment (Fig. 20A). This association correlated with observations made in transcription experiments. Cos7 cells transiently expressing C/EBPβ and the GR ligand binding domain were treated with dex in the presence or absence of proteasome inhibitor. In the absence of MG132, the GR ligand binding domain was able to potentiate C/EBPβ-mediated transcription by approximately 12-fold (Fig. 20B). Addition of MG132 resulted in a 3-fold decrease in transcription by the GR ligand binding domain, but had no effect on the steroid independent transcription.

C/EBPβ is acetylated following dex treatment.

The loss of C/EBPβ-associated HDAC1 from the C/EBPα promoter itself following dex treatment resulted in the acetylation of histone H4. Since C/EBPβ is associated with the HAT p300, it was hypothesized that C/EBPβ itself was a target of acetylation. Large scale (5 mg of cell extract) immunoprecipitations of C/EBPβ from 3T3 L1 cells induced to differentiate in the presence of MIX and insulin or MIX, insulin and dex for 24 hrs revealed an increase in acetylation following dex treatment (Fig. 21A). The acetylation was revealed using a pan-acetyl lysine antibody. Similar results were obtained from immunoprecipitates of C/EBPβ from Cos7 cells transiently expressing both C/EBPβ and the GR ligand binding domain (GR_{505C}), which indicates that the ligand binding domain of GR is sufficient to transduce the effects of steroid leading to the acetylation of C/EBPβ (Fig. 21B). The acetylation of C/EBPβ, therefore, correlates with the effects of dex on the potentiation of differentiation and on the C/EBPβ-HDAC1 complex.

Figure 20. MG132 treatment maintains HDAC1 at the C/EBPα promoter and represses transcription.

(A) Chromatin immunoprecipitation of HDAC1 at the C/EBPα promoter following 24 hr treatment with MIX and insulin (MI) in the absence or presence of dex and MG132 as indicated.

(B) Cos7 cells were transfected with 100 ng βgal, 200 ng of the C/EBPα reporter pCX14/12, 400 ng C/EBPβ and 100 ng GR_{505C} as indicated. Cells were treated with dex and MG132 for 24 hrs as indicated. Transcription from the C/EBPα promoter was measured by luciferase assay. Luciferase activity was corrected for transfection efficiency using βgal activity. Data is represented as fold induction by dex treatment. Error bars represent the standard error of the mean from three independent experiments performed in duplicate.

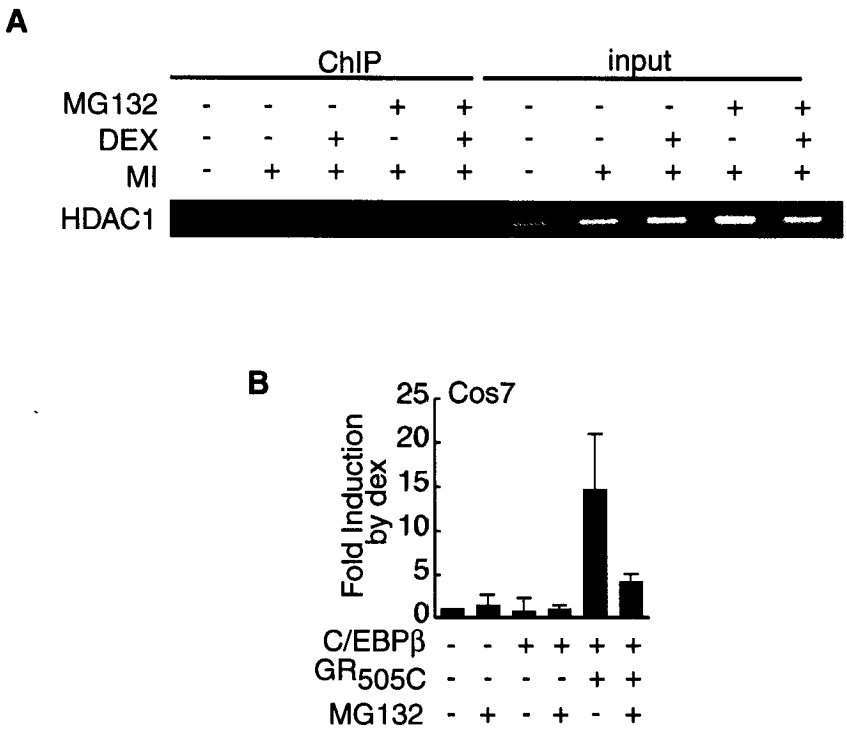
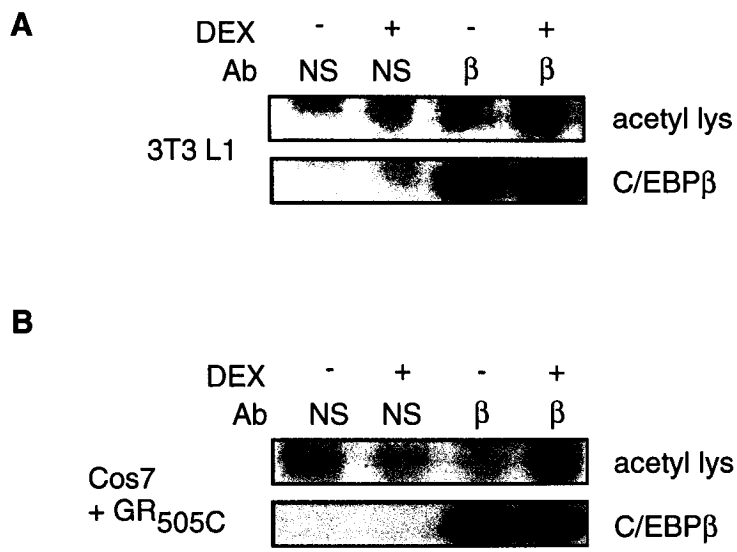

A
ChIP
input
MG132 - - - + + - - - + +
DEX - - + - + - - + - +
MI - + + + + - + + + +
HDAC1
B
Cos7
Fold Induction by dex
25
20
15
10
5
0
C/EBPβ - - + + + +
GR505C - - - - + +
MG132 - + - + - +

Figure 21. C/EBPβ is acetylated in vivo following dex treatment.

(A) Cell extracts (5 mg) from 3T3 L1 cells induced to differentiate with a 24 hr treatment of MIX, insulin or MIX, insulin and DEX were immunoprecipitated with anti-C/EBPβ antibody (β) or type-matched non-specific antibody (NS). Acetylation of immunoprecipitated C/EBPβ was detected using a pan-acetyl lysine antibody. The migration of C/EBPβ was detected using an anti-C/EBPβ antibody.

(B) Cell extracts from Cos7 cells transiently transfected to express C/EBPβ and the GR ligand binding domain (GR_{505C}) were treated with dex for 24 hr as indicated and immunoprecipitated as above.


A
DEX - + - +
Ab NS NS β β
3T3 L1
acetyl lys
C/EBPβ
B
DEX - + - +
Ab NS NS β β
Cos7
+ GR505C
acetyl lys
C/EBPβ

C/EBPβ is acetylated *in vitro* by GCN5 and PCAF.

To identify the acetylase responsible for acetylation of C/EBPβ, *in vitro* modification of GST-C/EBPβ was attempted with the candidate GCN5. The acetylase p300 has already been identified as a coactivator of C/EBPβ and has been observed associated with C/EBPβ by coimmunoprecipitation and by chromatin immunoprecipitation of the C/EBPα promoter. Recombinant p300 was used to attach ^{14}C-acetyl groups to the transcription factor but surprisingly was found to be unable to modify the protein, though it readily acetylated purified chicken histones (Fig. 22). In constrast, PCAF, a p300 associated histone acetyltransferase was found to be able to efficiently acetylate C/EBPβ *in vitro*. The closely related acetylase GCN5 was also found to acetylate C/EBPβ under these conditions.

PCAF and GCN5 are differentially expressed in mammalian tissues (Xu et al., 2000; Yamauchi et al., 2000). To determine which acetylases were expressed in our cell culture systems, whole cell extracts from these cells were analyzed by Western analysis for PCAF and GCN5 expression. Both 3T3 L1 and Cos7 cells express GCN5, though PCAF was undetectable in the preadipocytes (Fig. 23). HeLa nuclear extract are shown as a positive control for the Western blot.

C/EBPβ interacts *in vivo* with GCN5 and PCAF.

Coimmunoprecipitation experiments revealed that endogenous C/EBPβ interacted *in vivo* with GCN5 in 3T3 L1 preadipocytes. This interaction occured following hormonal induction of C/EBPβ protein with MIX and insulin, and was unaffected by the addition of dex (Fig. 24A). In a similar experiment, C/EBPβ and PCAF were transiently expressed in Cos7 cells. C/EBPβ efficiently coprecipitated PCAF both in the presence and absence of dex treatment (Fig. 24B).

Figure 22. C/EBPβ is acetylated in vitro by recombinant histone acetyltransferases PCAF and GCN5.

Bacterially expressed GST tagged C/EBPβ (GST-C/EBPβ), GST alone and purified chicken histones were in vitro acetylated with ^{14}C-acetyl CoA by 500ng of recombinant acetylases p300, PCAF and GCN5. Acetylation was detected by phosphorimager analysis.

A

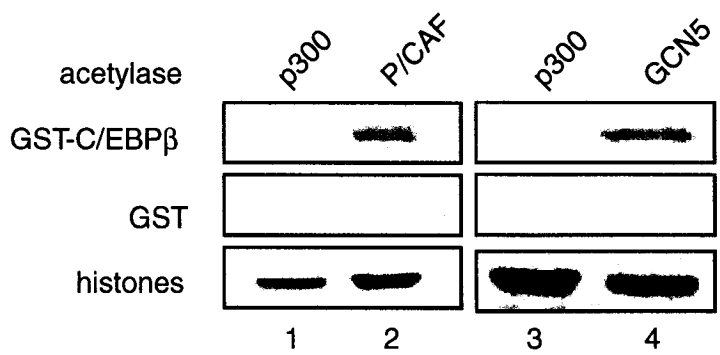


Figure 23. PCAF and GCN5 are differentially expressed in Cos7 and 3T3 L1 cells.
Western analysis of PCAF and GCN5 expression in Cos7 cells and 3T3 L1 preadipocytes. HeLa nuclear extract was used as a positive control.

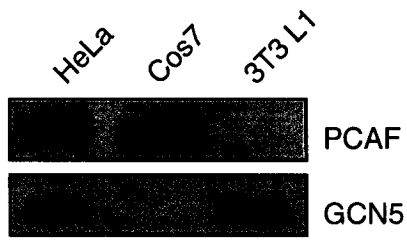
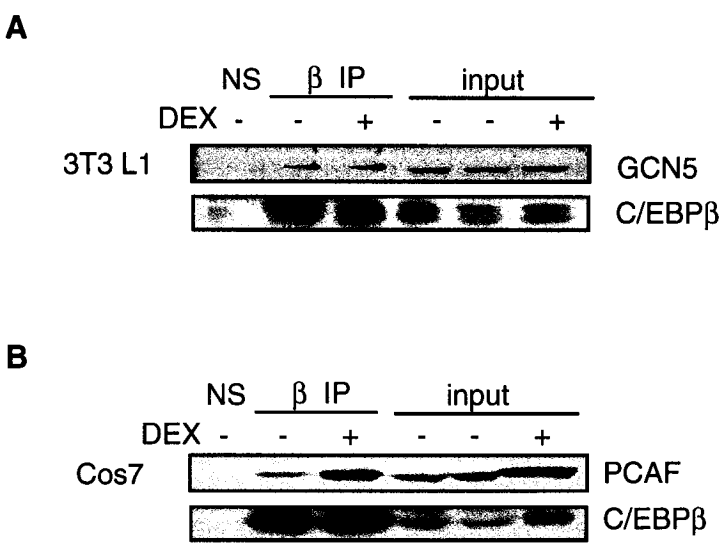

HeLa
Cos7
3T3 L1
PCAF
GCN5

Figure 24. Histone acetyltransferases GCN5 and PCAF interact with C/EBPβ in vivo.

(A) Endogenous GCN5 coimmunoprecipitated with C/EBPβ from 3T3 L1 extracts prepared after a 24 hr treatment with MIX and insulin in the presence or absence of dex as indicated. GCN5 and C/EBPβ were detected by Western analysis. Inputs represent 10% of the material used for immunoprecipitation.

(B) PCAF coimmunoprecipitates with transiently transfected C/EBPβ from Cos7 cells expressing the GR ligand binding domain (GR_{505C}) and treated with vehicle (-) or dex (+) for 24 hrs.


A
NS
β IP
input
DEX - - + - - +
3T3 L1
GCN5
C/EBPβ
B
NS
β IP
input
DEX - - + - - +
Cos7
PCAF
C/EBPβ

Human PCAF and full-length murine GCN5 are 87% identical at the amino acid level. In both mice and humans, a truncated form of GCN5 has been characterized and extensively used in experimentation. This form of GCN5 lacks the bulk of the N-terminus of the protein but retains the centrally located HAT domain, and the C-terminal bromodomain (Fig. 25A). Transfected C/EBPβ interacted with deletion constructs of human PCAF which lack portions of the HAT domain suggesting that this region is dispensable for the interaction (Fig. 25B). In these experiments, C/EBPβ interacted efficiently with the ΔHAT1 construct (deletion of amino acids 579-608) and with the ΔHAT2 construct (deletion of amino acids 609-624) (Fig. 25B).

In vitro pulldown experiments using GST-C/EBPβ revealed that C/EBPβ interacted with both the full length murine GCN5 and full-length human PCAF as expected based on the coimmunoprecipitation results (Fig 22C). In similar experiments, the short form of human GCN5 was equally capable of binding GST-C/EBPβ. These results suggest that the interaction between C/EBPβ and GCN5 (or PCAF) is direct, and reemphasizes that it does not require the N-terminus of the protein. In addition, both the ΔHAT1 and ΔHAT2 mutants of PCAF interacted *in vitro* with GST-C/EBPβ support the coimmunoprecipitation results suggesting this region to be dispensible for the interaction (Fig. 25C).

GCN5 is constitutively present at the C/EBPα promoter of 3T3 L1 preadipocytes.

Chromatin immunoprecipitation from 3T3 L1 cells induced to differentiate with MIX and insulin, or MIX, insulin and dex demonstrated the association of endogenous GCN5 with the C/EBPα promoter (Fig. 26). This association did not depend on the presence of C/EBPβ, as it was equally seen in cells treated with vehicle alone (-C), conditions where the C/EBPβ protein is not upregulated and where C/EBPβ is not seen associated with the promoter. The presence of this acetylase in the absence of DNA-bound C/EBPβ may explain the primed nature of the

Figure 25. C/EBPβ interacts with the C-terminus of GCN5.

(A) Schematic representation of the PCAF and GCN5 constructs used. Human PCAF and murine full length GCN5 (mGCN5) are of similar size and share 87% amino acid identity over the entire length of the proteins. Human GCN5 has a truncated N-terminus resulting in a 52 kDa protein. The PCAF HAT mutants ΔHAT1 and ΔHAT2 have deletions in the HAT domain which abolish enzymatic activity. The deletions are from amino acid 579-608 and 609-624 of the human protein respectively.

(B) Ectopic PCAFΔHAT1 and ΔHAT2 interact with transiently transfected C/EBPβ in Cos7 cells by coimmunoprecipitation. The amount of C/EBPβ in immunoprecipitates is shown by Western analysis using anti-C/EBPβ antibody.

(C) Association of ^{35}S-labelled GCN5 and PCAF constructs with GST-tagged C/EBPβ (GST-C/EBPβ) and GST alone as a control. In vitro translated luciferase is used as a control for the absence of binding. Inputs represent 10% of the radiolabelled protein used in the binding reaction.

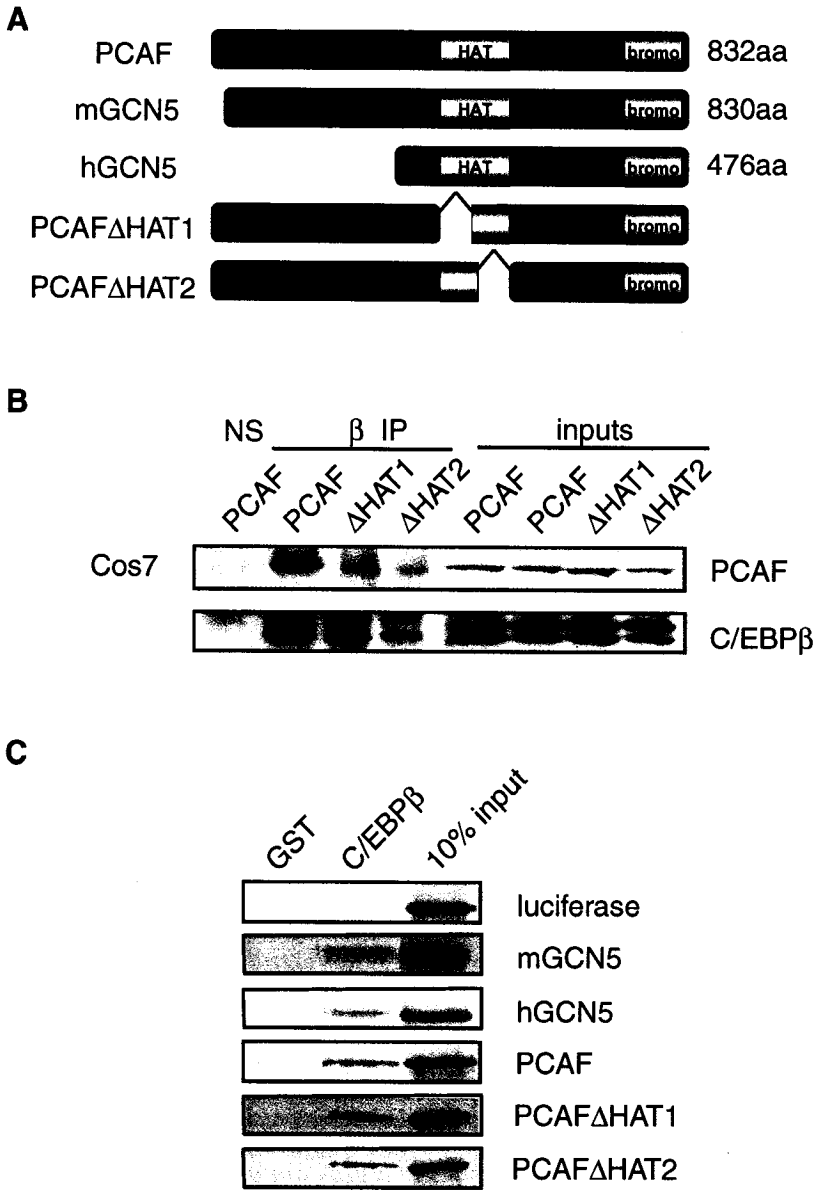

A
PCAF
HAT
bromo
832aa
mGCN5
HAT
bromo
830aa
hGCN5
HAT
bromo
476aa
PCAFΔHAT1
bromo
PCAFΔHAT2
bromo
B
NS
β IP
inputs
PCAF
PCAF
ΔHAT1
ΔHAT2
PCAF
PCAF
ΔHAT1
ΔHAT2
Cos7
PCAF
C/EBPβ
C
GST
C/EBPβ
10% input
luciferase
mGCN5
hGCN5
PCAF
PCAFΔHAT1
PCAFΔHAT2

Figure 26. GCN5 is found at the C/EBPα promoter of 3T3 L1 cells.

Chromatin immunoprecipitation of C/EBPβ and GCN5 crosslinked to the C/EBPα promoter in 3T3 L1 cells induced to differentiate for 24 hrs in the absence of cocktail (-C), or in the presence of MIX and insulin (+MI) or MIX, insulin and dex (+MID).

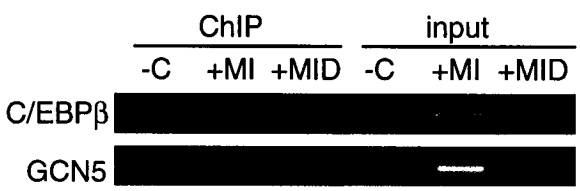

ChIP
input
-C
+MI
+MID
-C
+MI
+MID
C/EBPβ
GCN5

Table 1. Lysine clusters within transcriptional regulators shown to be acetylated. For each protein indicated, the acetylation site and the acetylase is indicated. C/EBPβ has been demonstrated to be acetylated upstream of the DNA-binding domain and within the activation domain in this work.

Protein	Acetylation motif	Identified Acetylase	Reference
C/EBPβ	AKPSKK , KAKK	PCAF, GCN5, CBP	Herein, Xu et al., 2003
ACTR (NCoA3)	SKGHKK	p300, CBP	Chen et al., 1999
Androgen receptor	KLKK	p300, PCAF	Fu et al., 2003
E2F	GKGVKSPEK	PCAF	Martinez -Balbas et al., 2000
MyoD	CKACKRK	PCAF, p300	Polesskaya et al., 2000; Sartorelli et al., 1999
p53	LKSKK	p300	Barlev et al., 2001; Gu and Roeder, 1997
GATA -1	GKGKKK, RPKKR	p300/CBP	Boyes et al., 1998

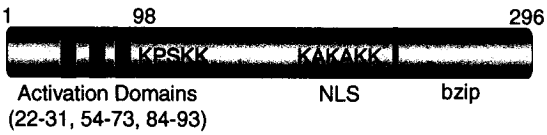
Figure 27. Mutation of lysines 98, 101, and 102 abolishes the acetylation of C/EBPβ detected in vivo.

(A) Schematic representation of murine C/EBPβ including the two lysine clusters. Lysines 98, 101 and 102 are located immediately downstream of the activation domains. The 2nd cluster is the nuclear localization signal (NLS) (top). The lysine cluster consisting of lysines 98, 101 and 102 are highly evolutionarily conserved (bottom).

(B) In vitro acetylation reactions of wild type GST-C/EBPβ and a mutant where lysines 98, 101 and 102 were replaced with arginine residues (K98-102R). Acetylation was performed as before with recombinant PCAF and incorporation of ^{14}C-acetyl CoA was detected by autoradiography and with the acetyl lysine antibody. Equal loading of GST-proteins is indicated by Coomassie staining.

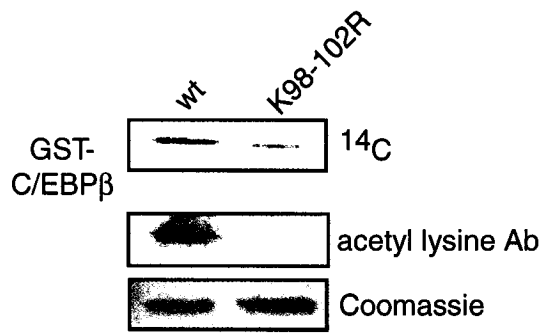
(C) Interaction of in vitro translated ^{35}S labeled human GCN5 with GST alone, GST-C/EBPβ and GST-C/EBPβK98-102R. Luciferase is shown as a negative control for binding. Inputs represent 10% of the radiolabelled protein used for the binding reaction

A

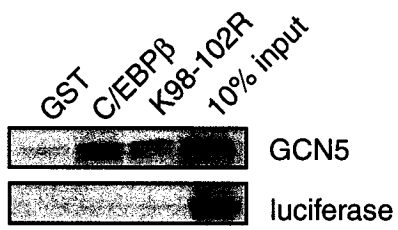


		identity
mouse	FADDYGA**K**PS**KK**PADYG	
rat	FADDYGA**K**PS**KK**P**S**DYG	94.1%
human	F**S**DDYG**GKNCKK**PA**E**YG	64.7%
bovine	F**S**DDYG**GKNCKKA**A**E**YG	55.6%

B



C



pan-acetyl lysine antibody used to detect the modification *in vivo*, a complete loss of signal from the C/EBPβK98-102R mutant was observed (Fig. 27B).

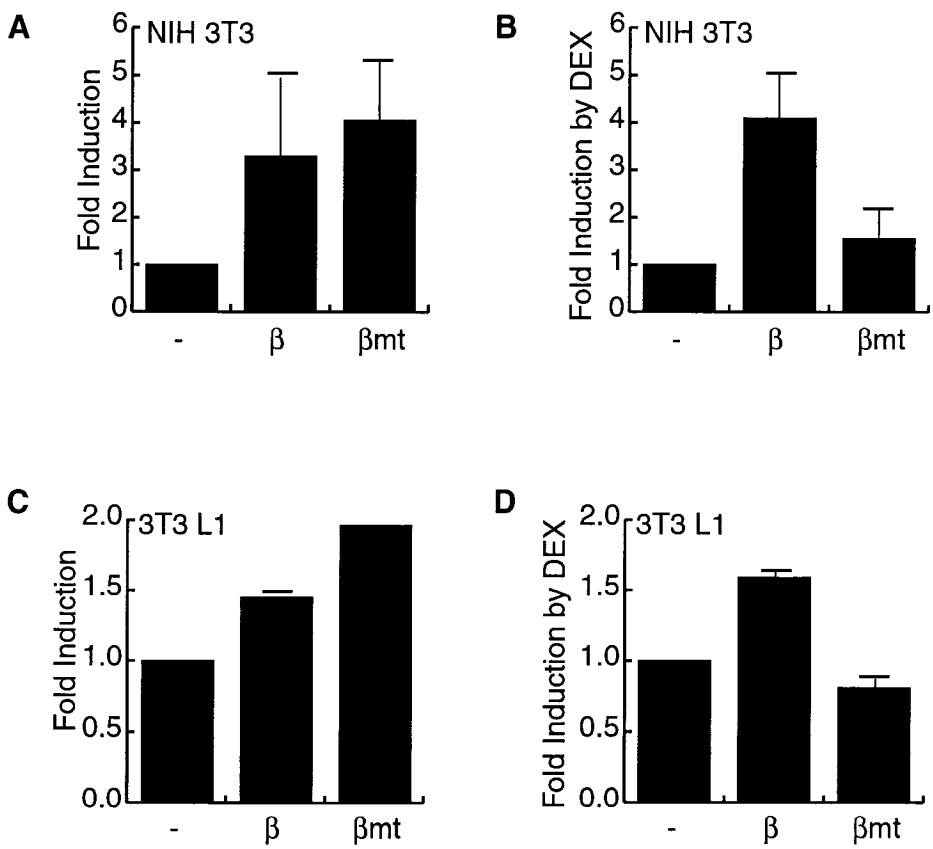
Despite the decrease in acetylation of the C/EBPβK98-102R mutant, it was still able to interact efficiently with radiolabelled human GCN5, in a manner comparable to the wild type C/EBPβ in a GST binding assay (Fig. 27C).

Acetylation within lysines 98, 101 and 102 increases the activation potential of C/EBPβ.

Acetylation of many transcription factors results in a modulation of their ability to activate transcription, often by influencing their ability to contact their cognate DNA binding site in promoter regions (Boyes et al., 1998; Gu and Roeder, 1997; Hung et al., 2001; Xu et al., 2003). Acetylation may also modulate their ability to interact with coregulatory molecules, thereby releasing corepressors or increasing association with coactivator complexes (Chen et al., 1999). Mutation of lysine 98, 101 and 102 of C/EBPβ did not alter its ability to activate the low level of transcription observed in the absence of dexamethasone treatment in transient transcription assays using the C/EBPα promoter (Fig. 28A). However, following dex treatment, the transcriptional activation from the C/EBPα promoter by the wild type C/EBPβ was greatly enhanced by approximately 4-fold (Fig. 28B). In contrast there was little enhancement of the acetylation mutant's transcriptional activity by the steroid treatment.

Similar results were obtained in 3T3 L1 preadipocytes. In the absence of dex treatment, transcription from the C/EBPα promoter by wild type C/EBPβ was enhanced by approximately 1.5-fold over basal promoter activity (Fig. 28C). The mutant was equally able to activate transcription in the absence of steroid. Following a 24 hr dex treatment, transcription by the wild type C/EBPβ increased by 2-fold whereas the mutant's activity was unaffected (Fig. 28D). These results suggest that acetylation within lysine residues 98, 101 and 102 is required to achieve the

Figure 28. Transcription by C/EBPβK98-102R is not potentiated by the glucocorticoid receptor.
(A) NIH 3T3 cells were transiently transfected with the C/EBPα reporter construct and the expression plasmids for C/EBPβ and the C/EBPβK98-102R (βmt). Transcription was measured 48 hrs after transfection by luciferase activity, and transfection efficiency was corrected for βgal activity. Data is represented as fold induction over basal activity. Data is representative of 3 independent experiments performed in duplicate. Error bars are the standard error of the mean.
(B) NIH 3T3 cells, transfected as in (A) were treated with dex 24 hrs after transfection for 24 hrs. Transcription was measured as in (A). Data is represented as fold induction by dex treatment, and is representative of 3 independent experiments performed in duplicate.
(C) Transient transcription experiment as in (A) performed in 3T3 L1 cells.
(D) Transient transcription experiment as in (B) performed in 3T3 L1 cells.


A
NIH 3T3
Fold Induction
0
1
2
3
4
5
6
-
β
βmt
B
NIH 3T3
Fold Induction by DEX
0
1
2
3
4
5
6
-
β
βmt
C
3T3 L1
Fold Induction
0.0
0.5
1.0
1.5
2.0
-
β
βmt
D
3T3 L1
Fold Induction by DEX
0.0
0.5
1.0
1.5
2.0
-
β
βmt

full transcriptional potential of C/EBPβ. The acetylation may serve to prevent the association of corepressor molecules such as mSin3A, or may participate in the stabilization or recruitment of coactivator molecules.

C/EBPβK98-102R remains associated with HDAC1 following dex treatment.

To evaluate the interaction of coregulatory molecules with the C/EBPβK98-102R mutant, coimmunoprecipitation experiments were performed in NIH 3T3 cells retrovirally expressing the wild type and mutant C/EBPβ constructs. Extracts were prepared following a 24 hr treatment of two-day post-confluent cells with MIX and insulin, or MIX, insulin and dex. As expected, endogenous GCN5 coimmunoprecipitated with both the wild type and mutant C/EBPβ with equal efficiencies (Fig. 29A). HDAC1 interacted with both the wild type and mutant constructs in the absence of dex treatment, but following steroid treatment the interaction between C/EBPβ and HDAC1 was strongly decreased. The interaction between C/EBPβK98-102R and HDAC1 was enhanced by treatment with dexamethasone.

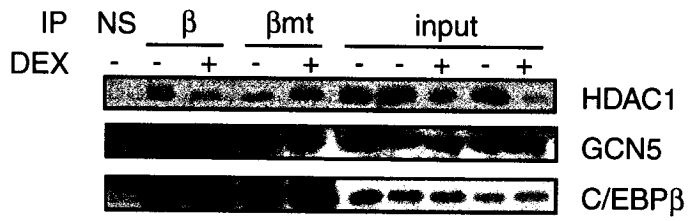
The association of the C/EBPβK98-102R mutant with HDAC1 following dex treatment was also observed on the C/EBPα promoter by chromatin immunoprecipitation (Fig. 29B). In the absence of dex treatment, HDAC1 was detected at the C/EBPα promoter regardless of the C/EBPβ construct expressed. Following dex treatment, the HDAC1 associated with the promoter in the presence of the wild type C/EBPβ was lost as expected, but HDAC1 was still seen associated with promoter in the presence of the mutant C/EBPβ. The association of the wild type and mutant C/EBPβ proteins with the promoter was comparable following 24 hr treatment with MIX and insulin, or MIX, insulin and dex.

Figure 29. Interaction with HDAC1 is maintained following dex treatment in cells expressing C/EBPβK98-102R.

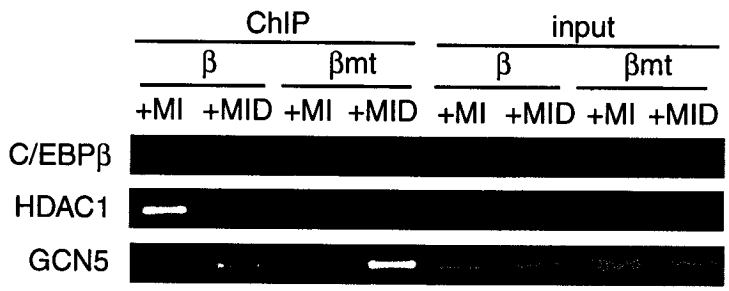
(A) Coimmunoprecipitation of HDAC1 and GCN5 with retrovirally expressed C/EBPβ or C/EBPβK98-102R (βmt) in NIH 3T3 cells. Cells were induced to differentiate for 24 hours with MIX and insulin in the presence (+) or absence (-) of dex as indicated, and immunoprecipitated with anti-C/EBPβ antibody or type-matched non-specific antibody (NS).

(B) Chromatin immunoprecipitation of C/EBPβ, HDAC1 and GCN5 from NIH 3T3 cells retrovirally expressing wild-type C/EBPβ or the K98-102R mutant (βmt) following a 24 hour treatment with MIX and insulin (+MI) or with MIX, insulin and dex (+MID).

A


IP
NS
β
βmt
input
DEX
- - + - + - - + - +
HDAC1
GCN5
C/EBPβ

B


ChIP
input
β
βmt
β
βmt
+MI +MID +MI +MID +MI +MID +MI +MID
C/EBPβ
HDAC1
GCN5

Mutation of lysines 98, 101 and 102 prevents maximal differentiation of NIH 3T3 and 3T3 L1 preadipocytes.

Consistent with the transient transcription results, expression of wild type C/EBPβ in NIH 3T3 cells that were allowed to differentiate for the full 8 days showed strong differentiation as revealed by Oil red O staining and an increase in adipsin expression (5.2±0.7 fold)(Fig. 30A), The expression of both C/EBPα and PPARγ, measured following 24 hours of hormone treatment, were also increased (3.4±0.1 fold and 7.4±0.3 fold respectively) (Fig. 30B). The K98-102R mutant, although still able to induce differentiation in these cells, was not able to do so to the same extent (approximately 50% reduction in stained cells, Fig. 30A) and after 24 hrs the levels of C/EBPα and PPARγ were lower than in cells expressing the wild type factor despite equal expression of the C/EBPβ constructs (7.7±0.5 fold for the wild type and 9.1±0.5 fold for the mutant) (Fig. 30B). C/EBPα expression was the same as in cells not expressing C/EBPβ (1.2±0.1 fold) and PPARγ was only induced 3.0±0.1 fold. Thus, in cells expressing the C/EBPβ K98-102R mutant, the levels of C/EBPα were reduced by 3-fold and the levels of PPARγ were reduced 2.5-fold compared to cells expressing the wild-type C/EBPβ.

Forced expression of C/EBPβ in 3T3 L1 preadipocytes results in differentiation of the cells in the absence of any hormonal stimulation. In these cells, expression of wild-type C/EBPβ resulted in strong differentiation of the cells as revealed by Oil red O staining and adipsin levels (Fig. 30C). Adipsin protein levels were increased by 5.7±0.2 fold. Expression of both C/EBPα and PPARγ were induced after 24 hours (3.2±0.3 fold and 4.7±0.3 fold respectively) (Fig. 30D). In parallel to the results obtained in NIH 3T3 cells, the mutated C/EBPβ was less able to drive differentiation despite comparable expression to the wild type (Fig. 30D), and differentiated cells had decreased expression of C/EBPα (2.1±0.6 fold), PPARγ (1.0±0.1 fold) and adipsin (0.9±0.1 fold) (Fig 30C and D).

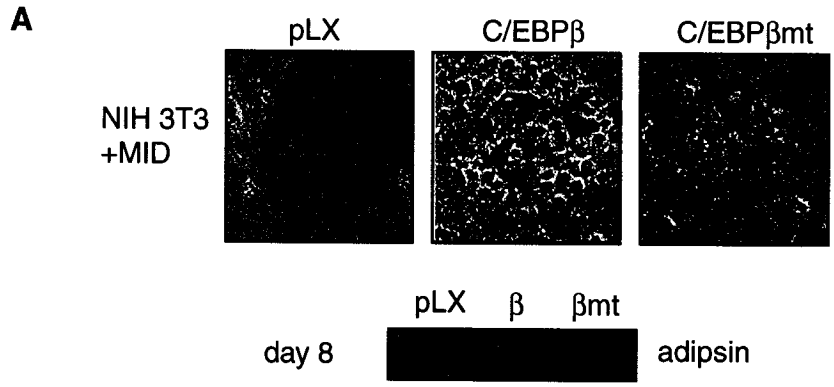
Figure 30. Retroviral expression of C/EBPβK98-102R reduces the differentiation of NIH 3T3 cells and 3T3 L1 preadipocytes.

(A) NIH 3T3 cells retrovirally expressing wild-type C/EBPβ or the K98-102R mutant (C/EBPβmt) were induced to differentiate with MIX, insulin and dex. Eight days post-induction, the cells were stained with Oil red O to reveal neutral lipid droplets (top) and harvested for Western analysis of adipsin (bottom).

(B) Cells induced to differentiate as in (A) were harvested 24 hours post-induction for Western analysis of C/EBPβ expression, as well as the differentiation markers C/EBPα and PPARγ.

(C) 3T3 L1 cells retrovirally expressing wild-type C/EBPβ or the K98-102R mutant (C/EBPβmt) were allowed to spontaneously differentiate in growth medium for 8 days following confluency. Cells were stained with Oil red O (top) and harvested for Western analysis of adipsin protein (bottom).

(D) Cells induced to differentiate as in (C) were harvested 24 hours post-induction for Western analysis of C/EBPβ expression, as well as the differentiation markers C/EBPα and PPARγ.


A
pLX
C/EBPβ
C/EBPβmt
NIH 3T3
+MID
pLX
β
βmt
day 8
adipsin
B
pLX
β
βmt
24 hr
C/EBPβ
C/EBPα
PPARγ
C
pLX
C/EBPβ
C/EBPβmt
3T3 L1
pLX
β
βmt
day 8
adipsin
D
pLX
β
βmt
24 hr
C/EBPβ
C/EBPα
PPARγ

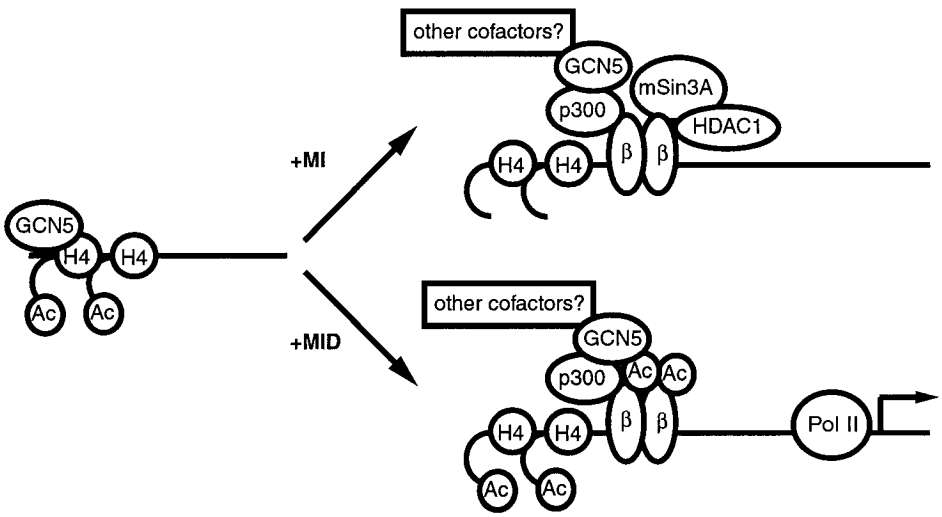
Transcription from the C/EBPα promoter is controlled by the balance of associated coactivators and corepressors.

The results indicate a mechanism whereby C/EBPβ is associated with p300, GCN5 and with an mSin3A/HDAC1 complex (Fig. 31). Upon treatment with dexamethasone, the HDAC1 is targeted for degradation by the GR ligand binding domain, and the association between C/EBPβ and mSin3A is lost. The histone acetyltransferases p300 and GCN5 remain associated with C/EBPβ under these conditions, tilting the balance of coactivators and corepressors in the favour of acetylation. In the absence of HDAC1, both histone H4 at the C/EBPα promoter and C/EBPβ itself are acetylated. Acetylated C/EBPβ cannot interact with HDAC1 and is thereby more transcriptionally potent. Active transcription is confirmed by the interaction of RNA polymerase II at the promoter.

Figure 31. Acetylation of C/EBPβ by GCN5 correlates with increased transcriptional potential on the C/EBPα promoter.

In the absence of dex treatment, C/EBPβ is associated with coactivator molecules p300 and GCN5 in 3T3 L1 preadipocytes. C/EBPβ also interacts with a repressive complex composed of HDAC1 and mSin3A. The corepressor complex prevents activation of transcription through reduced histone H4 acetylation and concomitant lack of RNA polymerase II recruitment. Upon dex treatment, the HDAC1 component of the corepressor complex is degraded and histone H4 and C/EBPβ itself are acetylated. Acetylation of C/EBPβ correlates with increased transcription from the C/EBPα promoter and the modification may prevent the interaction of C/EBPβ with other repressive HDAC1 containing complexes.

C/EBPα promoter



DISCUSSION

Cushing's syndrome, or hypercortisolism, can result from the overproduction of cortisol or from pharmacological use of glucocorticoids. The common sign of this disease is obesity that is resistant to weight loss strategies. Patients treated with valproic acid, a powerful anti-convulsant and mood stabilizer, risk similar obesity during treatment. The results of this thesis suggest that the obesity seen in Cushing's syndrome and in patients treated with valproic acid is the result of a convergent molecular pathway involving the development of new adipocytes from precursor cells. The data shows that the glucocorticoid receptor acts to relieve transcriptional repression by HDAC1 that acts as a blockade against preadipocyte differentiation. Direct inhibition of HDAC1 activity by valproic acid's histone deacetylase inhibitor activity equally relieves transcriptional repression and results in the development of new adipocytes from precursor cells.

On a molecular level, this thesis provides evidence that the GR and the progesterone receptor ligand binding domains can enhance the transcription mediated by DNA-bound C/EBPβ through the targeted degradation of an HDAC1-containing complex. This is the first report of an activating non-transcriptional role for these steroid receptors. Furthermore, evidence is presented indicating that C/EBPβ itself is a target for acetylation, and that this acetylation may serve as not only a marker for the loss of associated HDAC1, but also as a modification which prevents the interaction of C/EBPβ with other minor C/EBPβ-associated HDAC1-containing complexes. This acetylation is required for full preadipocyte differentiation potential in both 3T3 L1 preadipocytes and NIH 3T3 fibroblasts, and correlates with increased transcription from the C/EBPα promoter in transient transfection assays.

In the absence of steroid treatment, C/EBPβ, the histone acetyltransferase p300 and an HDAC1 and mSin3A-containing complex appear to segregate into a discrete complex by FPLC.

The interactions between p300 and C/EBPβ and C/EBPβ and the HDAC1 complex appear to be separate, as coimmunoprecipitation of HDAC1 did not coprecipitate detectable p300. Thus, C/EBPβ exists in a dynamic equilibrium between interactions with activating acetylases and the HDAC1-containing complex. Steroid treatment causes the targeted degradation of the HDAC1-containing complex without impacting the interaction of C/EBPβ with p300. Under these conditions, the promoter architecture is changed by acetylation of promoter-associated histone H4 allowing the recruitment of RNA polymerase II to the C/EBPα promoter. These changes reflect the transcriptional status of the promoter and indicate that C/EBPβ is able to activate transcription. The equilibrium shift in favour of acetylase activity on the C/EBPα promoter following steroid treatment is also reflected by the acetylation status of C/EBPβ itself. This acetylation, when prevented by mutation of the critical lysines 98, 101 and 102 to arginine residues results in a sustained interaction with HDAC1 despite the loss of the identified C/EBPβ-associated HDAC1-containing complex. This persistent association with HDAC1 prevents full activation of C/EBPα transcription by the glucocorticoid receptor and results in minimal preadipocyte differentiation.

The GR ligand binding domain acts to potentiate C/EBPα transcription and preadipocyte differentiation.

The agonist-bound glucocorticoid receptor has been characterized to function in three principal ways: by binding to its DNA-response elements in promoter regions to activate transcription through the recruitment of histone acetyltransferase molecules and chromatin remodeling complexes, by binding DNA-response elements and repressing transcription through an unknown mechanism (so called negative GREs) (Drouin et al., 1987; Drouin et al., 1989) and by the interference or crosstalk with other DNA-bound transcription factors possibly through

coactivator theft, as in the case of AP-1 (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990) and NF-kB (Almawi and Melemedjian, 2002). Here, evidence is presented that supports a new role for the agonist-bound glucocorticoid receptor. In this circumstance, the receptor acts via a non-transcriptional mechanism, and activates transcription indirectly by targeting for degradation a C/EBPβ-interacting HDAC1-containing complex. This effect of the receptor requires only the ligand binding domain. The degradation of HDAC1 by the 26 S proteasome is responsible for the release of C/EBPβ from the repressive action of the deacetylase on the C/EBPα promoter, a phenomenon that correlates with an increase in C/EBPα transcription and greatly enhanced preadipocyte differentiation in cell culture models. Steroid treatment was unable to enhance C/EBPβ-mediated transcription from the PPARγ promoter. On this promoter, C/EBPβ was incapable of driving transcription despite being able to bind the response elements and glucocorticoid receptor activation had no further effect. Chromatin immunoprecipitations recently performed in our laboratory reveal that in 3T3 L1 cells treated with MIX and insulin or MIX, insulin and dex, C/EBPβ does bind the PPARγ promoter with equal efficiency (D. Wu, personal communication). On this promoter, C/EBPβ is associated with HDAC1 even after treatment with dex, unlike the C/EBPα promoter. This sustained interaction may explain why C/EBPβ is not able to activate transcription from the PPARγ promoter in the transient transcription experiments. The question remains why the C/EBPβ-associated HDAC1 is not affected by steroid treatment on this promoter. One possibility is the promoter context itself. The PPARγ promoter is controlled by two tandem consensus C/EBP motifs whereas the C/EBPα promoter is controlled by one degenerate motif. Perhaps the C/EBPβ-HDAC1 complex that associates with the PPARγ promoter is in fact different from the complex described for the C/EBPα promoter. Indeed, the association of cofactors such as GCN5 is critical in ensuring the full transcriptional activation by C/EBPβ via acetylation on the C/EBPα promoter, and may be lacking on the PPARγ promoter.

Degradation of the Sin3 subcomplex is a property of the progesterone receptor ligand binding domain.

The progesterone receptor ligand binding domain was able to enhance C/EBPβ-mediated transcription of the C/EBPα promoter to the same extent as the glucocorticoid receptor. Recapitulation of the effects of dexamethasone by steroid treatment suggests that the degradation of the HDAC1-containing complex is a conserved mechanism among steroid hormone receptors. However, on synthetic promoter templates the androgen receptor was not able to potentiate C/EBPβ-mediated transcription. In fact, the effect of androgens was repressive by up to 2-fold (Boruk et al., 1998). These results are in agreement with the clinical application of androgens, which increase muscle mass without increasing adiposity (Singh et al., 2003). Thus, the androgen receptor, and perhaps other steroid receptors, may not share the HDAC1-reducing property of the glucocorticoid and progesterone receptors. The androgen receptor LBD is very similar to the GR LBD on an amino acid level, but appears to function differently. In fact, the AF-2 of the androgen receptor is weak in comparison to other steroid receptors and appears to contribute very little to transactivation (Jenster et al., 1991). Coactivators such as SRC-1, which interact with GR and the progesterone receptor AF-2 interact with the androgen receptor via AF-1 (Bevan et al., 1999; He et al., 1999). Furthermore, the androgen receptor AF-1 interacts with the AF-2, and this interaction appears to block interaction of coactivators with this domain (He et al., 2001). This type of interaction between the N and C-termini has not been described for GR or other steroid receptors. The differences in the coregulator interaction surface and in AF-1/AF-2 interaction may not allow the androgen receptor to function as the GR LBD in the targeting of HDAC1 to the proteasome.

Progestins were equally capable of driving preadipocyte differentiation in 3T3 L1 preadipocytes that retrovirally expressed the progesterone ligand binding domain. The

recapitulation of the glucocorticoid effect by progestins in this system indicates that the receptor ligand binding domain is sufficient to transmit the effects of steroid. Western analysis from cell extracts produced from cells treated with progestins indicates that following hormone treatment, the HDAC1 protein levels decrease by approximately 50% and led to the dissociation of C/EBPβ from mSin3A/HDAC1, as was seen with glucocorticoid treatment.

In Cos7 cells, which lack endogenous glucocorticoid receptor, the degradation of HDAC1, both from endogenous and exogenous source, occurred when the GR ligand binding domain was expressed and agonist was present. In essence, the activation of the endogenous full-length glucocorticoid receptor is not necessary to achieve the HDAC1 reducing effects. These results are in accord with the GR^{dim} mice whose glucocorticoid receptor lacks DNA-binding ability (Reichardt et al., 1998). These mice are of normal weight, consistent with the effects of glucocorticoids on preadipocyte differentiation being mediated through a process independent of DNA binding.

Treatment of 3T3 L1 preadipocytes with the histone deacetylase inhibitors trichostatin A or valproic acid also enhanced differentiation, though addition of dex to this treatment had no further effect. In fact, in transient transfection assays using the C/EBPα promoter, treatment with TSA greatly enhanced transcription mediated by C/EBPβ but addition of dex to the deacetylase inhibitor treatment did not further potentiate transcription. These results indicate that the histone deacetylase inhibitors and the effect of steroid converge on the same pathway. Inhibition of the C/EBPβ-associated HDAC1 by the inhibitors allows the environment around C/EBPβ to be more favorable to acetylases, as reflected by the increase in C/EPBPα transcription and protein, and enhanced preadipocyte differentiation. Treatment with steroid leads to a targeted degradation of the C/EBPβ-associated population of HDAC1 and thereby frees C/EBPβ from its repressive effects. Since the end result of both mechanisms is the removal of HDAC1's deacetylase

capacity and results in maximal transcription from the C/EBPα promoter, treatment with both histone deacetylase inhibitors and steroid together cannot have synergistic effects.

The valproic acid used in these experiments has only recently been identified as a histone deacetylase inhibitor (Gottlicher et al., 2001; Phiel et al., 2001). It has, however, been used extensively pharmacologically as a treatment for both epilepsy and mood disorders (Ernst and Goldberg, 2003; Vajda, 2002). One of the frequently reported side effects of this drug is weight gain (Davis et al., 1994; Jallon and Picard, 2001). These reports support the model presented here, where inhibition of HDAC1 by valproic acid would free C/EBPβ to activate transcription of C/EBPα and to thereby drive preadipocyte differentiation, resulting in an increase in adiposity.

Identification of the HDAC1-containing complex by FPLC.

HDAC1 has been shown to exist in many complexes including the nucleosome remodeling and deacetylase complex (NuRD), the CoREST complex and the Sin3 complex (Ayer, 1999; You et al., 2001). These complexes differ not only in their protein components but also in their roles in transcription. The NuRD complex is essential for embryonic patterning, Wnt signaling, Hox expression (Chen and Han, 2001) and regulation of Ras signaling (Solari and Ahringer, 2000; von Zelewsky et al., 2000) in *Drosophila* and *C. elegans*. The complex contains HDAC1, HDAC2, the chromatin binding proteins RbAp46 and 48, as well as the ATP-dependent nucleosome remodeling protein Mi-2 and MTA1/2 which are overexpressed in metastatic carcinomas (Calvo et al., 2002; Mahoney et al., 2002). The CoREST complex was identified as a novel complex that lacked RbAp48 and RbAp46. It does however include both HDAC1 and HDAC2, as well CoREST (You et al., 2001). To date, the role of this complex in transcriptional regulation has been limited to the silencing of neuronal genes activated by REST (Battaglioli et al., 2002). The Sin3 complex, so named because it includes mSin3A as a proposed scaffold protein for the complex, has been implicated in repression mediated by unliganded nuclear

hormone receptors, in particular the retinoic acid and thyroid hormone receptors (Heinzel et al., 1997) and in cell cycle progression in *Drosophila* (Pile et al., 2002). Corepressors NCoR and SMRT link the Sin3 complex to the nuclear hormone receptor and may play a direct role in transcriptional repression independent of HDACs (Wong and Privalsky, 1998a).

The size exclusion chromatographic separation of HDAC1-containing complexes in 3T3 L1 cells revealed 6 distinct peaks. These peaks differed in their HDAC1 content as well as in their composition of mSin3A and RbAp48. One complex, peak VI was lost following a 24 hr treatment with dexamethasone. Peak VI included approximately one third of the cellular HDAC1 and half of the cellular C/EBPβ. This peak contained mSin3A but lacked RbAp48, consistent with the proteins identifed to interact with C/EBPβ by coimmunoprecipitation. This Sin3 sub-complex differs from previously identified HDAC1-containing complexes in that it specifically lacks HDAC2. The lack of both HDAC2 and the Rb-associated proteins suggests that this complex functions differently from the Sin3 complex to repress transcription. The Rb-associated proteins have been shown to act as chromatin binders and may serve to target deacetylase complexes to chromatin, directing their enzymatic activity to acetylated histones (Verreault et al., 1998; Zhang et al., 2000).

Dex treatment of 3T3 L1 cells also resulted in a decrease in the HDAC1 content of peak V without any change in molecular weight. Therefore, the protein complex that elutes as peak V is not altered in composition but it is reduced in the cell following steroid treatment. Peak IV, upon dex treatment, elutes at a later fraction than in the absence of steroid, indicating that the complex is of smaller molecular weight. This result suggests that a protein or proteins are lost from the peak IV complex upon dex treatment. Quantification of the FPLC data obtained from extracts treated with dex reveal that in fact the mSin3A content of peak IVa is significantly reduced in comparison to peak IV (Appendix D). The shift in molecular weight could then represent, at least in part, the loss of mSin3A from this complex. Interestingly, the C/EBPβ

content of this peak doubles following steroid treatment, suggesting the existence of another C/EBPβ-HDAC1 complex, which in this case does not include mSin3A. Since the *in vitro* binding assays demonstrate that HDAC1 does not directly interact with C/EBPβ in an mSin3A-free complex other proteins must act as a bridge between C/EBPβ and HDAC1. In fact, the interaction of the C/EBPβK98-102R mutant with HDAC1 but not mSin3A following dex treatment provides evidence that HDAC1 may interact with C/EBPβ through a protein other than mSin3A.

The other components of peak VI Sin3 subcomplex, if any, have yet to be identified. GST pulldown experiments suggest that C/EBPβ interacts directly with mSin3A but not with HDAC1 and thus mSin3A may serve as an anchor point for HDAC1 association with C/EBPβ. However, despite many descriptions of HDAC1-mSin3A coassociation (Hassig et al., 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang et al., 1997), a direct demonstration of interaction between these two proteins is lacking. Consequently, other unidentified proteins may exist to bridge mSin3A and HDAC1 in this complex. The domains of C/EBPβ and mSin3A required for their interaction have not been elucidated.

C/EBPβ exists in a dynamic equilibrium with p300 and HDAC1.

The results of coimmunoprecipitation experiments presented herein indicate that C/EBPβ is found to interact with both p300 and HDAC1-mSin3A. However, control immunoprecipitations of HDAC1 do not coprecipitate p300, suggesting that C/EBPβ exists in a dynamic equilibrium between these two opposing factors, which interact with C/EBPβ separately. The histone acetyltransferase p300 has been shown to interact with C/EBPβ through the first 21 amino acids of the protein (Mink et al., 1997). This region has also been implicated in the association of the ATP-dependent chromatin remodeling complex Swi/Snf (Kowenz-Leutz and

Leutz, 1999). Although it is unknown which surface of C/EBPβ is required for interaction with the deacetylase complex, it is possible that p300 and mSin3A-HDAC1 bind the same site on C/EBPβ. Human Swi/Snf complexes containing Brahma (hBrm) and brm/swi-related gene-1 (Brg-1) have been shown to interact with members of the Sin3 corepressor complex (Sif et al., 2001). In particular, hBrm can interact with mSin3A, HDAC1, HDAC2 and RbAp48. The interaction of hBrm with the C/EBPβ N-terminus could also act as an interaction surface for mSin3A (Kowenz-Leutz and Leutz, 1999). Thus, interaction of the Swi/Snf complex alone would lead to coactivation of C/EBPβ's transcriptional potential whereas association of an hBrm-mSin3A-HDAC1 complex would act as a repressor.

Treatment with steroid and the degradation of the associated deacetylase complex leaves C/EBPβ to interact with p300 as reflected by increased histone H4 acetylation, the recruitment of RNA polymerase II to the C/EBPα TATA box and enhanced transcription. The equilibrium thereby shifts in favour of the acetylase, and the full transcriptional potential of C/EBPβ is realized.

The role of GR in HDAC1 metabolism.

Results presented herein demonstrate that dexamethasone treatment reduces HDAC1 protein levels by approximately 50% and completely ablates the HDAC1 content of a C/EBPβ-interacting complex. Coimmunoprecipitation experiments show that liganded GR interacts with both HDAC1 and mSin3A at 4 hrs of steroid treatment. GR is not seen to interact with RbAp48 or HDAC2 under these conditions, suggesting that it does interact with the same complex that interacts and represses C/EBPβ. Mechanistically there exist at least two possibilities explaining the role of GR in removing HDAC1 from C/EBPβ. First, it is possible that GR-HDAC1/mSin3A and C/EBPβ form a ternary complex. GR would then interact with HDAC1/mSin3A when bound

to C/EBPβ and remove or inactivate the corepressors from the transcription factor. This type of mechanism has been suggested to account for the repression of AP-1-mediated transcription by glucocorticoids (Yang-Yen et al., 1990). The second possibility is the titration of the HDAC1-containing complex by liganded GR. In this scenario, both GR and C/EBPβ are able to interact with the HDAC1-containing complex. Upon steroid treatment and nuclear localization of GR, the receptor binds to the HDAC1-containing complex. Proteasomal degradation of the complex then reduces the amount of HDAC1 available in the cell to bind C/EBPβ.

In these experiments, it was observed that short steroid treatments of 4 hrs have no impact on HDAC1 protein levels. Furthermore, the interaction of GR with HDAC1 and mSin3A does not influence the interaction between the corepressors and C/EBPβ. In fact, it was not possible to detect GR associated with the C/EBPα promoter by chromatin immunoprecipitation. Given that detection of a ternary complex has not been possible the results favour the second of the above two possible mechanisms. The data presented herein support independent interactions between GR and HDAC1 and C/EBPβ and HDAC1.

Targeting of HDAC1 to the 26S proteasome by the GR ligand binding domain.

Many nuclear hormone receptors have been observed to be degraded by the 26 S proteasome following ligand activation (Boudjelal et al., 2002; Lange et al., 2000; Masuyama and MacDonald, 1998; Nawaz et al., 1999a; Wallace and Cidlowski, 2001). It is believed that this property of nuclear receptors may serve to attenuate the transcriptional response mediated by the receptors. In particular, GR has been shown to be both ubiquitylated and degraded by the 26 S proteasome by Cidlowski (Wallace and Cidlowski, 2001). The results presented herein illustrate a more complex portrait of GR turnover than that observed by Wallace and Cidlowski. In these experiments, GR is observed to be strongly and rapidly degraded following hormone treatment, and protein levels are almost obliterated after a 24 hr steroid treatment. These results are

consistent with published results of Cidlowski and others (Deroo et al., 2002; Wallace and Cidlowski, 2001). In contrast to Wallace and Cidlowski where treatment with proteasome inhibitors completely stabilized receptor levels, addition of the proteasome inhibitor MG132 only partially rescues the receptor from proteasomal degradation with the bulk of the receptor population still lost. The discrepancy from published data may be the result of the overexpression system used by Wallace and Cidlowski. The experimental results discussed here are based on observation of the endogenous receptor in 3T3 L1 preadipocytes. The physiological levels studied in these experiments eliminate the possible contribution of a misfolded protein response due to massive overexpression of the receptor. Unlike Wallace and Cidlowski, higher molecular weight forms of GR were not observed following MG132 treatment. This may be due to the small percentage of the receptor that is processed by the proteasome in the experiments discussed herein, making detection of this subpopulation technically challenging.

At first glance the HDAC1 protein turnover does not follow the same kinetics of degradation as GR. The turnover of HDAC1, which is completely blocked by the proteasome inhibitor MG132, is much slower than that of GR. Whereas GR is completely degraded following a 24 hr dexamethasone treatment, HDAC1 levels are only reduced by half. However, the FPLC data demonstrates that only a specific subpopulation of HDAC1 is sensitive to steroid induced degradation, and thus all of the HDAC1 within peak VI is lost. Consequently, the kinetics do support the possibility that destruction-bound GR may act to transport HDAC1 to the proteasome.

Given that only a small proportion of GR is observed to be degraded by the proteasome, it is unlikely that HDAC1 represents an accidental passenger to be degraded. In fact, since higher order forms of HDAC1 are observed, which may represent ubiquitylation, it is more likely that GR-associated HDAC1 (HDAC1 from peak VI) is actively tagged for destruction by enzymes of the ubiquitin pathway. GR has been demonstrated to interact with E3 ubiquitin ligases, enzymes responsible for the conjugation of activated ubiquitin onto substrates (Nawaz et al., 1999b;

Sengupta and Wasylyk, 2001). Interaction of GR with HDAC1 may bring the deacetylase substrate into close proximity with an E3 ubiquitin ligase, thereby facilitating HDAC1's tagging for destruction. In particular, GR has been demonstrated to accelerate p53 degradation through a trimeric complex with the E3 ligase Mdm2, while being tagged for destruction itself (Sengupta and Wasylyk, 2001). In normal endothelial cells under hypoxic conditions and in HepG2 cells under normoxia, GR, p53 and the human homolog of Mdm2, Hdm2, form a ligand dependent trimeric complex in the cytoplasm of cells, leading to the ubiquitylation and degradation of both GR and p53. Mdm2 has been shown to interact with HDAC1, and this interaction leads to deacetylation of p53, a step required for its degradation by the proteasome (Ito et al., 2002), providing possible preliminary evidence that these two proteins can form an enzyme-substrate complex. During preadipocyte differentiation of 3T3 L1 cells, p53 mRNA and protein levels steadily decrease as does p53's DNA binding activity (Berberich et al., 1999). Since acetylation of p53 is associated with an increase in DNA binding ability, it is possible that deacetylation by Mdm2-associated HDAC1 results in degradation of the p53 protein. Decreasing p53 protein levels would then effectively shut down p53 mRNA production, as p53 regulates its own promoter (Deffie et al., 1993). HDAC1 itself, in the context of glucocorticoid treatment may also be a substrate for this E3 ligase.

O'Malley's laboratory has identified E6-AP, a ubiquitin ligase first characterized as the gene responsible for Angelman's syndrome, as a coactivator molecule for the progesterone receptor and GR as well as for androgen and estrogen receptors (Nawaz et al., 1999b). The E3 ubiquitin ligase activity of E6-AP is dispensable for activation of steroid receptor-mediated transcription, but it remains possible that the interaction of liganded GR and the HDAC1-containing complex specifically tags the HDAC1 for degradation. An interaction between HDAC1 and E6-AP has not been described.

Finally, C/EBPβ has been shown to interact with, and be activated by, the RING-finger protein TIF1β (Chang et al., 1998). Although TIF1β has not been identified as an E3 ligase, it

possesses a RING finger domain that is characteristic of a subclass of E3 ligases that includes Mdm2. The coactivation of C/EBPβ by TIF1β is consistent with a role for TIF1β in the ubiquitylation of HDAC1. Overexpression of the E3 ubiquitin ligase could then accelerate the turnover of HDAC1, resulting in enhanced transcription by C/EBPβ. Liganded GR has also been reported to interact with TIF1β, an interaction that leads to coactivation of GRE-dependent transcription by the receptor. On the α1-acid glycoprotein gene, a promoter controlled by both C/EBP elements and a GRE, ectopic TIF1β can enhance transcription from this promoter even after mutation of the GRE (Chang et al., 1998). Mutation of the C/EBP motifs ablates the effect of TIF1β. These results are consistent with a role for TIF1β in the targeting of GR-associated HDAC1 for degradation by the 26S proteasome.

Interestingly, mSin3A protein levels are not affected by treatment with dexamethasone. While liganded GR interacts with mSin3A and HDAC1, only HDAC1 and a small percentage of GR is lost to the proteasome. Mechanistically, the absence of evident mSin3A turnover suggests two possibilities. First, the peak VI HDAC1-containing complex associates with liganded GR where it is brought into close proximity with a GR-associated E3 ubiquitin ligase. Recognition of the HDAC1 substrate by the E3 ligase results in the formation of ubiquitin chains on HDAC1. The modification of HDAC1 may dissociate the GR-mSin3A-HDAC1 complex, freeing the HDAC1 to move to the proteasome without the other complex components. Alternatively, ubiquitylated HDAC1 may remain associated with GR (which may also be modified by the E3 ubiquitin ligase) and the whole complex is brought to the proteasome. Since the specificity of ubiquitin conjugation is determined by the E3 ubiquitin ligase, only the components of the complex tagged for destruction would pass through the proteasome. The mSin3A content would then be spared from destruction.

The dynamic equilibrium surrounding C/EBPβ is reflected in its acetylation status.

Following treatment with steroid, when histone H4 is found to be acetylated and transcription from the C/EBPα promoter is maximal, an increase in C/EBPβ acetylation is also observed using a pan-acetyl lysine antibody. Unexpectedly, p300 did not acetylate C/EBPβ *in vitro*. In contrast, the p300 associated acetylase PCAF, and the closely related GCN5 were found to efficiently acetylate C/EBPβ under the same conditions. In 3T3 L1 preadipocytes only GCN5 was detected, suggesting that GCN5 may be a physiologically relevant acetylase in preadipocyte differentiation that acts through C/EBPβ. Mouse knockout studies have shown that PCAF is dispensable, but loss of GCN5 is embryonic lethal (Xu et al., 2000; Yamauchi et al., 2000). GCN5 null embryos show severe growth defects and fail to form dorsal mesoderm. Knockout of both PCAF and GCN5 results in more severe defects and suggests that although GCN5 functions in mammalian development are more important, there is likely functional overlap between the two acetylases in tissues where they are coexpressed.

The ability of C/EBPβ to interact both *in vivo* and *in vitro* with PCAF and GCN5 reflects the highly conserved natures of their sequences. GCN5 and PCAF are approximately 87% identical on the amino acid level. They are used interchangeably in many *in vitro* experiments. The relative importance of one versus the other may be dictated by their tissue distribution and interactions with other activating complexes or transcription factors.

The interaction of GCN5 with C/EBPβ was not affected by treatment with dexamethasone. In fact, the recruitment of GCN5 to the C/EBPα promoter was independent of C/EBPβ binding to its response element as seen in chromatin immunoprecipitation experiments in cells that were not induced to differentiate. This association with the promoter in the absence of C/EBPβ correlates with the acetylation of histone H4 in untreated cells. The acetylation in the absence of C/EBPβ binding may reflect a primed state for the C/EBPα promoter in 3T3 L1 preadipocytes, and may be reflective of the commitment of the preadipocytes to the adipocyte

lineage. In murine fibroblasts that are not committed to the adipocyte lineage, such as NIH 3T3 or SF7 fibroblasts, the acetylation of H4 is not observed. In fact, recent chromatin immunoprecipitation experiments have shown that GCN5 is absent from the C/EBPα promoter in NIH 3T3 cells that are not expressing C/EBPβ (D. Wu, personal communication). It is then possible that DNA-associated GCN5 may be responsible for the acetylation status of histone H4 at the C/EBPα promoter in 3T3 L1 preadipocytes. Although experimental results presented here are insufficient to demonstrate this possibility, targeted knockdown of GCN5 in these cells, perhaps by RNA interference, could answer the requirement for GCN5 for differentiation and H4 acetylation.

The presence of GCN5 at the promoter in the absence of C/EBPβ binding raises another important question. If C/EBPβ is not responsible for the recruitment of its own acetylase, how is GCN5 targeted to the promoter? The primers used for chromatin immunoprecipitation assays flank the C/EBP motif of the C/EBPα promoter. This region, spanning from –334 to –118 base pairs includes the C/EBP motif at –197 to –178 base pairs, as well as putative sites for upstream stimulating factor (USF), NF-kB and a degenerate NF1-like site (Legraverend et al., 1993). Neither NF-kB nor NF1 have been shown to interact with GCN5 or GCN5-containing complexes. However, the binding of c-Myc to the upstream stimulating factor site could impact the acetylation status of the promoter. c-Myc has been shown to interact with TRRAP-GCN5 complexes and this interaction is thought to play a role in chromatin opening through acetylation of histones (Flinn et al., 2002; Liu et al., 2003). If this mechanism is at play in 3T3 L1 preadipocytes, c-Myc is not involved in transcriptional activation of the C/EBPα gene in the absence of C/EBPβ. Treatment with MIX and insulin results in upregulation of C/EBPβ protein levels and its binding to the C/EBP motif of the promoter. The binding of C/EBPβ to the promoter puts the transcription factor in close proximity to GCN5, perhaps forging a protein-protein interaction as suggested by the results of coimmunoprecipitation and GST pulldown

experiments. The presence of both c-myc and C/EBPβ on the promoter would form a cooperative complex where, following treatment with steroid, the recruited GCN5 could acetylate C/EBPβ.

Acetylation of C/EBPβ limits association with HDAC1.

The interaction and colocalization of HDAC1 and the C/EBPβK98-102R mutant following steroid treatment suggests that C/EBPβ can interact with other HDAC1-containing complexes. The results from the size exclusion chromatographic separation indicate that many minor C/EBPβ-containing complexes (containing from 10-17% of cellular C/EBPβ) exist in the cell (peaks I, III, IV and V). Western analysis of HDAC1 protein levels following dex treatment in NIH 3T3 cells retrovirally expressing the C/EBPβ acetylation mutant show a decrease in HDAC1 levels comparable to that seen in cells expressing the wild type C/EBPβ (Fig. 29A inputs). These results suggest that the HDAC1 of peak VI is likely still degraded, though confirming FLPC data is lacking.

The interaction of HDAC1 with the C/EBPβK98-102R mutant following dex treatment may then occur by the following mechanism. Upon loss of peak VI, the wild type C/EBPβ is acetylated by GCN5 preventing any further interaction with HDAC1 from another source. In the case of the mutant, modification by acetylation cannot occur, leaving the mutant C/EBPβ free to interact with minor HDAC1-containing complexes. This new association prevents the full transcriptional potential of C/EBPβ by maintaining the equilibrium between associated HATs and HDAC1 in the favour of the deacetylase. Recent experiments indicate that mSin3A no longer interacts with the wild type or mutant C/EBPβ by coimmunoprecipitation following dex treatment. The corepressor mSin3A is also dissociated from the C/EBPα promoter under these conditions. Thus, the interaction of the C/EBPβK98-102R mutant with HDAC1 in the absence of mSin3A supports a model where a new HDAC1-containing complex, which specifically lacks

mSin3A, associates following dex treatment. Since C/EBPβ is not degraded by the proteasome following dex treatment, it is predicted that the proportion of C/EBPβ found in peak VI would be redistributed following dex treatment. Further analysis of the FPLC data indicates that the C/EBPβ content of peak III is unchanged after dex treatment (Appendix D). However, approximately 20% of the C/EBPβ can now be detected in a high molecular weight complex comigrating with HDAC1 peak II. The C/EBPβ content of peak IVa and peak V doubles and triples respectively. The mSin3A is also redistributed, and is found to be more diffuse through the HDAC1 peaks. It remains absent however in peak V, and interestingly is lost from peak IVa. These results suggest that the C/EBPβK98-102R mutant may associate with a redistributed peak V or peak IVa complex.

Biological impact of C/EBPβ acetylation.

Association of C/EBPβ with the HDAC1-containing complex results in minimal transcriptional activation of the C/EBPα promoter and low levels of adipocyte differentiation. Following dex treatment, the HDAC1 complex is lost and C/EBPβ is acetylated likely by GCN5. Acetylation is dominant under these conditions, histone H4 is modified and RNA polymerase II is recruited to the promoter. The C/EBPβK98-102R mutant is not acetylated following dex treatment and remains associated with HDAC1. Thus, minimal transcriptional potential is observed following dex treatment as the acetylation mutant is kept in a repressed state by HDAC1. These observations are made in transient transfection assays using the C/EBPα promoter, and in adipocyte differentiation where the C/EBPβK98-102R mutant can only drive minimal differentiation, and low levels of C/EBPα protein are detected. Release of C/EBPβ from HDAC1 is imperative for full activation of C/EBPα transcription and therefore for efficient preadipocyte differentiation.

Biological implications beyond C/EBPα and adipocyte differentiation

The enhancement of transcription by the glucocorticoid and progesterone receptor ligand binding domains through degradation of HDAC1 is physiologically important. The receptor ligand binding domain is sufficient to conduct the actions of glucocorticoids in the differentiation of preadipocytes. The ability of the progesterone receptor to substitute for the glucocorticoid receptor in these experiments suggests that the regulation of HDAC1 protein levels is a property that may be shared by steroid receptor ligand binding domains. The progesterone receptor, by virtue of its differential tissue expression could influence C/EBPβ–mediated transcription in biological systems where glucocorticoid action is absent. For example, progesterones are very active in the ovary and the breast. Progesterone may act to enhance C/EBPβ-mediated transcription pathways involved in ovulation and in lobuloalveolar development of the breast during pregnancy (Seagroves et al., 2000; Sterneck et al., 1997).

These experiments examine the role of the GR ligand binding domain-mediated degradation of HDAC1 in C/EBPβ-driven preadipocyte differentiation. However, it is likely that the downregulation of HDAC1 by steroid treatment also influences the action of other transcription factors. In particular, the C/EBPβ-interacting complex of HDAC1 could interact with other transcription factors of the bzip family, or even other unrelated transcription factors. Furthermore, among the HDAC1 complexes identified by size exclusion chromatography peak V was also reduced in HDAC1 content. This complex may act on a different subset of transcription factors, or directly on chromatin to repress transcription. A new subset of genes would then also be indirectly regulated by steroid hormone treatment.

In preliminary work not described in the Results section, RNA harvested from 3T3 L1 cells retrovirally expressing the PR ligand binding domain were treated with MIX and insulin or MIX, insulin and R5020 and was used for microarray (Appendix E). Using an Affymetrix chip, the regulation of 10 000 murine genes was examined following a 4 hr, 36 hr and 48 hr treatment

with R5020. RNA was also collected from cells treated with MIX, insulin and the histone deacetylase inhibitor trichostatin A for 24 hrs to use as comparison, as well as cells treated with MIX, insulin and dex. From these preliminary experiments, 84 genes were found to be regulated by both dexamethasone and R5020 in this system. The 13 genes regulated by a 4 hr steroid treatment are unlikely to be regulated by the mechanism described herein. Short hormone treatments do not impact the HDAC1 protein levels or the interaction between C/EBPβ and HDAC1. These genes represent targets of the progesterone and glucocorticoid receptor ligand binding domain acting through a different mechanism, perhaps through the recruitment of coactivating molecules to DNA-bound transcription factors. It should be noted that both upregulation and downregulation of gene targets were observed, suggesting that the receptor ligand binding domain can not only act to enhance transcription, but may participate in its repression as well. In fact, gene-targeted mice expressing a DNA dimerization mutation which impairs DNA binding demonstrates the ability to repress AP-1 mediated transcription (Reichardt et al., 2000), though this particular transcriptional interference requires determinants in the DNA-binding domain. Nonetheless, a similar mechanism involving the GR LBD may be at play for some of the genes identified by microarray analysis. The 13 genes upregulated by a 36 hr steroid treatment do not overlap with genes identified at 4 hours, and may be regulated by the mechanism described in this work. After a 36 hr steroid treatment, the HDAC1 content of peak VI is ablated, and genes held in a repressed state by the deacetylase complex are transcribed. Not all of these genes have been identified as C/EBPβ-regulated genes. These genes can then serve to study the role of peak VI in transcription beyond C/EBPβ. It is interesting to note that another 6 genes were downregulated following a 36 hr steroid treatment. The mechanism of transcriptional downregulation in this case is unknown. A total of 40 genes were identified as being up or downregulated by a 48 hr steroid treatment. There exists some overlap (6 genes) between these genes and those identified at 36 hrs. Genes upregulated after 48 hrs of steroid treatment may also

represent targets of peak VI repression. Significant overlap is observed between genes regulated by dex and R5020 and those regulated by TSA treatment. The deacetylase inhibitor treatment should relieve repression by HDACs and the regulated genes should in part overlap with the genes regulated by the dex and R5020-dependent degradation of HDAC1. These genes represent promoters that should be regulated by the mechanism similar to that described in this thesis.

Future Directions

The higher molecular weight forms of HDAC1 seen upon proteasome inhibitor treatment are consistent with ubiquitylation. However, to confirm the ubiquitylation of HDAC1, it should be coexpressed with a tagged ubiquitin construct allowing for the immunoprecipitation of ubiquitylated HDAC1 from cells following dex and MG132 treatment. Once ubiquitylation has been confirmed, the modified lysine residues can be mapped using deletion constructs of HDAC1 and immunoprecipitation as before. It is predicted that an HDAC1 point mutant that can no longer be ubiquitylated will be insensitive to dex treatment and will repress C/EBPβ-mediated transcription from the C/EBPα promoter even in the presence of steroid treatment.

To identify the components of peak VI an FPLC separation would have to performed to obtain sample for identification by microsequencing. A similar approach could be used to identify the E3 ligase responsible for HDAC1 ubiquitylation. Although many candidates have been suggested herein, FPLC separation of HDAC1 containing complexes after a 4 hr dex treatment would reveal a GR/HDAC1 containing complex like the one observed by coimmunoprecipitation. The protein components of this complex could be identified by microsequencing allowing not only the identification of an E3 ubiquitin ligase (if any) but the conclusion that the GR LBD interacts with the same HDAC1 complex as C/EBPβ (peak VI).

Overexpression of the identified E3 ubiquitin ligase in the preadipocyte differentiation system should enhance differentiation in the absence of dex treatment. RNA interference

experiments in the preadipocyte where expression of the E3 ubiquitin ligase is diminished would differentiate very poorly, as it would be predicted that the HDAC1 levels would not diminish during the time frame of C/EBPβ action.

FPLC separation of the HDAC1 complexes associated with the C/EBPβ acetylation mutant will provide some interesting information about the regulation of C/EBPβ's transcriptional activity by GCN5. The C/EBPβ acetylation mutant could be expressed in NIH 3T3 cells and the HDAC1 complexes could be compared in the presence and absence of dex treatment. Preliminary evidence suggests that the C/EBPβ acetylation mutant will associate with a different population of HDAC1 than the wild-type C/EBPβ.

Although GCN5 is able to acetylate C/EBPβ *in vitro*, data presented in this thesis does not demonstrate a definitive role for GCN5 in differentiation. Overexpression of GCN5 in preadipocytes should enhance transcription of C/EBPα in early differentiation, and result in an increase in mature adipocytes as the balance between the positive acting GCN5 and the negative regulator HDAC1 changes. If RNA interference experiments designed to knock down GCN5 in preadipocytes result in a decrease in the observed differentiation, it would suggest that GCN5 is an important component of the early transcriptional cascade.

Concluding remarks

This thesis provides evidence that the steroid receptor ligand binding domains of the glucocorticoid receptor and the progesterone receptor can enhance C/EBPβ-mediated transcription from the C/EBPα promoter resulting in increased preadipocyte differentiation. This potentiation of transcription is a direct consequence of the targeted degradation of a specific C/EBPβ-interacting HDAC1-mSin3A complex by the 26 S proteasome. Degradation of HDAC1 frees DNA-bound C/EBPβ from its repressive effects on chromatin, and permits C/EBPβ itself to

be acetylated by promoter associated GCN5. Acetylation, which occurs within lysines 98, 101 and 102 of C/EBPβ prevents interaction of C/EBPβ with other HDAC1 containing complexes that differ in composition from the original repressive complex.

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APPENDICES

APPENDIX A : Cloning Strategies

Table 1. Plasmids obtained from other researchers.

Plasmid name	Type of plasmid	Description	Source	Reference
6RGR	Mammalian expression	Plasmid driving the expression of full length rat glucocorticoid receptor	Dr. K Yamamoto	(Pearce and Yamamoto, 1993)
C478	Reporter	Reporter construct containing –609 to +52 of the murine PPARγ promoter upstream of luciferase	Dr. J. Gimble	(Clarke et al., 1997)
pCDNA3.1-HDAC1	Mammalian expression	Plasmid driving the expression of full length human HDAC1 with a C-terminal HA and FLAG tags	Dr. X-J Yang (McGill)	(Yang et al., 1996a)
pCDNA3.1-mSin3a	Mammalian expression	Plasmid driving the expression of human mSin3a. This plasmid can be *in vitro* translated using a T7 primer	Dr. X-.J. Yang	unpublished
pCX14/12	Reporter	Reporter construct containing the –350 to +7 murine C/EBPα promoter upstream of luciferase	Dr. P. Antonsen	(Legraverend et al., 1993)
pMSVC/EBPα	Mammalian expression	Plasmid driving the expression of rat C/EBPα	Dr. S.K. McKnight	(Cao et al., 1991)
pMSVC/EBPβ	Mammalian expression	Plasmid driving the expression of murine C/EBPβ	Dr. S.K. McKnight	(Cao et al., 1991)
pMSVC/EBPδ	Mammalian expression	Plasmid driving the expression of murine C/EBPδ	Dr. S.K. McKnight	(Cao et al., 1991)
pT7hPRβ	*In vitro* translation	Plasmid encoding full length human progesterone receptor β	Dr. B.W. O'Malley	(Allan et al., 1992)
PTLrGR	Mammalian expression/IVT	Plasmid driving the expression of the full length rat glucocorticoid receptor	Joanne Savory	(Savory et al., 2001)

pCI-PCAF	Mammalian expression/IVT	Plasmid driving the expression of human PCAF	Dr. X-J Yang	(Yang et al., 1996b)
pCI-PCAFΔHAT1	Mammalian expression/IVT	Plasmid driving the expression of human PCAF with an internal deletion of amino acids 579-608	Dr. X-J Yang	(Yang et al., 1996b)
pCI-PCAFΔHAT2	Mammalian expression/IVT	Plasmid driving the expression of human PCAF with an internal deletion of amino acids 609-624	Dr. X-J Yang	(Yang et al., 1996b)
pFLAG-hGCN5	Mammalian expression	Plasmid driving the expression of FLAg-tagged human GCN5	Dr. S. Khochbin	(Col et al., 2001)
pHIS-hGCN5	Bacterial expression	Plasmid driving expression of human GCN5 in bacteria for purification using the 6X His tag.	Dr. S. Khochbin	(Col et al., 2001)
pmyc-hGCN5	*In vitro* translation	Plasmid encoding a myc-tagged human GCN5.	Dr. S. Khochbin	(Col et al., 2001)
pmmGCN5	Mammalian expression/IVT	Plasmid driving the expression of full-length murine GCN5.	Dr. S. Roth	(Xu et al., 1998b)

Table 2. Common vectors used for cloning

Vector Name	Vector Type	Mammalian Promoter	*In vitro* Translation	Source
pLXSN	Retroviral vector	Moloney murine Leukemia virus 5' LTR	No	Clontech
PTL2	Mammalian expression	SV40 early	Yes T7	M.Petkovich (Queen's University)
pTLmyc	Mammalian expression	SV40 Early	Yes T7	M.Petkovich (Queen's University)
PCR3.1-	Mammalian expression	CMV	Yes T7	Stratagene

Table 3. Strategies used for cloning using restriction enzyme digestion

Plasmid name	Type of plasmid	Description	Vector	Vector Digestion	Insert Origin	Insert Digestion
pLXSNGR	Retroviral expression	Retroviral vector containing full length rat glucocorticoid receptor	pLXSN	BamHI	6RGR	BamHI
pLXSNHDAC1	Retroviral expression	Retroviral vector containing full length human HDAC1	pLXSN	HpaI	PCDNA3.1-HDAC1	PmeI

Table 4. Strategies used for cloning using PCR amplification of the insert

Plasmid name	Type of plasmid	Description	Vector	Vector Digestion	Insert Origin	Primers for Amplification
pGEX2T HDAC1	GST fusion	Expresses a fusion protein of GST and full length human HDAC1 Constructed by Louise Pope	PGEX 2T	EcoRI	pCR3.1-HDAC1	F: 5'ctaggaaatcatatggcgcagacgcagggc3' R:5'acgagaattccagtcgaggctgatcagcgg3'
pGEX2T C/EBPβ	GST fusion	Expresses a fusion protein of GST and full length mouse C/EBPβ Constructed by Louise Pope	PGEX 2T	BamHI EcoRI	pMSV C/EBPβ	F: 5'actcggatccatgcaccgcctgctgggctg3' R:5'acgcgaattctagcagtggcccgccgaggc3'
pLXSN C/EBPβ	Retroviral expression	Retroviral vector containing C/EBPβ	pLXSN	EcoRI XhoI	PMSV C/EBPβ	F: 5'cgcccgaattccatgcaccgcctgctggcc3' R:5'ggggcgctcgagctagcagtggcccgccga3'
pLXSN C/EBPβK 98-102R	Retroviral expression	Retroviral vector containing the lys98, 101, 102 to arginine acetylation mutant of C/EBPβ	pLXSN	EcoRI XhoI	PMSV C/EBPβ K98-102R	F: 5'cgcccgaattccatgcaccgcctgctggcc3' R:5'ggggcgctcgagctagcagtggcccgccga3'
PLXSN mycGR$_{505C}$	Retroviral expression	Retroviral vector containing rat glucocorticoid receptor amino acids 505 to 795	pLXSN	XhoI BamHI	6RGR	F: 5'cgcggatcctaagactcactatag3' R: 5'cgcggatcctcatttttgatgaaacag3'
pLXSNmyc PR$_{632C}$	Retroviral	Retroviral vector containing a C-terminal myc tagged human progesterone receptor amino acids 632-930	pLXSN	BamHI	PTlmyc PR$_{632C}$	F: 5'cgcggatcctaagactcactatag3' R: 5'gccggatcctcacttttatgaaagag3'
PTLmyc GR$_{505C}$	Mammalian expression	Drives the expression of a C-terminal myc tagged rat glucocorticoid receptor amino acids 505 to 795	pTLmyc	XhoI BamHI	6RGR	F: 5'ccgctcgagatgaaccttgaagctcga3' R: 5'cgcggatcctcatttttgatgaaacag3'
PTLmyc PR$_{632C}$	Mammalian expression	Expressed N-terminal myc tagged human progesterone receptor amino acids 632 to 930	pTLmyc	XhoI BamHI	pT7hPRβ	F: 5' ccgctcgagatggtccttggaggg3' R: 5'gccggatcctcacttttatgaaagag3'
pTLRARα	Mammalian expression	Drives the expression of full length mouse RARα	pTL	XhoI BamHI	PGEM RARα	F:5'ggccggctcgagatggccagcaatagcagt3' R: 5'gcgggatcctcaggccttggcgctgatgct3'

Table 5. Stategies used for site-directed mutagenesis

Plasmid name	Parent Vector	Type of Plasmid	Description	Mutagenesis Primers	Unique Restriction site
pCXDmut	pCX14/12	Reporter	C/EBPα promoter cloned in front of luciferase	5'ggttcgccttgcagcgcaggagatcttcgcactcgagccac gatctctctcc3'	BglII
pCXLDE	pCXDmut	Reporter	C/EBPα promoter containing a partial mutation cloned in front of luciferase	5'cagcgcaggagatcttcgcacaggtagctcgagtgctctcc a3'	XhoI
pGEX2T C/EBPβ K98-102R	pGEX2T C/EBPβ	GST fusion	For expression of a GST-C/EBPβ fusion protein bearing a lysine to arginine mutation at lysines 98, 101, 102 (acetylation mutant) Constructed by Mei Wu	5'gacgactacggcgccaggccgtctagaaggccggccgact acggt3'	XbaI
pMSV C/EBPβ K98-102R	pMSV C/EBPβ	Mammalian expression	Drives expression of the murine full length C/EBPβ with an acetylation mutation at lysines 98, 101, and 102 (converted to arginine)	5'gacgactacggcgccaggccgtctagaaggccggccgact acggt3'	XbaI

APPENDIX B: Chemical Suppliers

Ligands and inhibitors

Product	Chemical name	Supplier
All-trans retinoic acid	3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol	Steraloids (Newport, Rhode Island)
Dexamethasone	1,4-pregnadien-9α-fluor-16α methyl-11β, 17, 21-triol-3-20-dione	Steraloids (Newport, Rhode Island)
MG132	N-CBZ-LEU-LEU-LEU-AL	Invitrogen (Burlington, Ontario)
Promegasterone (R5020)	17α-methyl-17-propionylestra-4,9-dien-3-one	NEN (Boston, Massachusetts)
RU486	RU 38486	Roussel-UCLAF (Paris, France)
Trichostatin A	4,6-dimethyl-7-[p-dimethylaminophenyl]-7-oxohepta-2,4,-dienohydroxamic acid	Sigma (Oakville, Ontario)
Valproic acid	2-propylpentanoic acid	Sigma (Oakville, Ontario)

Cell culture reagents

Reagent	Supplier
3-isobutyl-1-methylxanthine (MIX)	Sigma (Oakville, Ontario)
Calf serum	Invitrogen (Burlington, Ontario)
Dulbecco's Modified Eagle Medium	Invitrogen (Burlington, Ontario)
ExGen 500	MBI Fermentas (Burlington, Ontario)
Fetal bovine serum	Wisent (St.-Bruno, Quebec)
Fetal bovine serum	Invitrogen (Burlington, Ontario)
G418 (Geneticin)	Invitrogen (Burlington, Ontario)
Insulin	Sigma (Oakville, Ontario)
Lipofectamine	Invitrogen (Burlington, Ontario)
OptiMem	Invitrogen (Burlington, Ontario)

Commercial kits

Reagent	Supplier
Bradford reagent	Bio-Rad (Mississauga, Ontario)
Luciferase assay reagent	Promega (Madison, Wisconsin)
Ni-NTA Spin Kit	Qiagen (Mississauga, Ontario)
ONPG	Sigma (Oakville, Ontario)
Pfu mutagenesis kit	Stratagene (La Jolla, California)
Qiaquick PCR purification kit	Qiagen (Mississauga, Ontario)
Reporter lysis buffer	Promega (Madison, Wisconsin)
TNT T7 Coupled Reticulocyte System	Promega (Madison, Wisconsin)

Chemicals

Chemical	Supplier
1,4-dithiothreitol (DTT)	Sigma (Oakville, Ontario)
^{14}C-acetyl CoA	Amersham (Baie D'Urfe, Quebec)
2-mercaptoethanol	EM Science (Gibbstown, New Jersey)
^{35}S-methionine	Amersham (Baie D'Urfe, Quebec)
Chloroquine	Sigma (Oakville, Ontario)
Complete protease inhibitor	Roche (Laval, Quebec)
Core histones	Upstate Biotech (Charlottesville, Virginia)
Dihydroxyacetone phosphate	Sigma (Oakville, Ontario)
Dimethylpimedilate	Pierce – MJS BioLynx (Brockville, Ontario)
Ethanolamine	Sigma (Oakville, Ontario)
Formalin	BDH – VWR International (Mississauga, Ontario)
Glutathione sepharose beads	Amersham (Baie D'Urfe, Quebec)
IPTG	Sigma (Oakville, Ontario)
molybdate	Sigma (Oakville, Ontario)
Nonindet P40 (NP-40)	EM Science (Gibbstown, New Jersey)
Oil Red O	Sigma (Oakville, Ontario)
o-nitrophenyl β-D-galactopyronoside	Sigma (Oakville, Ontario)
Polybrene	Sigma (Oakville, Ontario)
Protein A	Sigma (Oakville, Ontario)
Protein G	Sigma (Oakville, Ontario)
PVDF membrane	Bio-Rad (Mississauga, Ontario)
Recombinant p300	Novagen – EMD Biosciences (Gibbstown, New Jersey)
Recombinant PCAF	Upstate Biotech (Charlottesville, Virginia)
Sephacryl s300 HR	Amersham (Baie D'Urfe, Quebec)
Sheared salmon sperm DNA	Ambion (Austin, Texas)
Sodium azide	Sigma (Oakville, Ontario)
Sodium borate	Sigma (Oakville, Ontario)
Sodium deoxycholate	EM Science (Gibbstown, New Jersey)
Thimerisol	Sigma (Oakville, Ontario)
Triton x-100	EM Science (Gibbstown, New Jersey)
Tween 20	EM Science (Gibbstown, New Jersey)
β-NADH	Sigma (Oakville, Ontario)

APPENDIX C : Abbreviations

C/EBP: CCAAT/enhancer binding protein

Dex: dexamethasone

FBS: fetal bovine serum

GR: glucocorticoid receptor

GRE: glucocorticoid response element

HAT: Histone acetyltransferase

HDAC: Histone deacetylase

LBD: ligand binding domain

MIX: methylisobutylxanthine

PBS: phosphate buffered saline

TSA: trichostatin A

VPA: valproic acid

APPENDIX D : Supplemental data

Fraction	I	II	III	IV	IVa	V	VI	
+MI	17	-	12	12	-	10	49	%C/EBPβ
+MID	-	22	11	-	28	30	-	%C/EBPβ
+MI	-	29	-	22	-	-	48	%mSin3A
+MID	16	25	37	-	3	6	13	%mSin3A

Table 1. Redistribution of C/EBPβ and mSin3A following dex treatment.

Six different HDAC1-containing complexes were observed in 3T3 L1 extracts prepared following a 24 hr treatment with MIX and insulin (+MI) or MIX, insulin and dex (+MID) when separated by size exclusion chromatography (See Fig 17B). Western blotting and phosphorimager analysis determined the relative distribution of C/EBPβ and mSin3A in each peak. Data is represented as a percent of total C/EBPβ protein (%C/EBPβ) or percent of total mSin3A (%mSin3A).

APPENDIX E : Supplemental data

Microarray results

All data presented in this appendix is the result of three independent experiments. The genes shown in the Tables 1-3 are regulated by R5020 in 3T3 L1 cells expressing the progesterone receptor LBD at the given time point, and are also regulated by treatment with dexamethasone. The Table 4 lists genes regulated by a 36 hr treatment with the histone deacetylase inhibitor TSA. Microarrays were performed in collaboration with Dr. Rob Sladek of the Montreal Genome Centre.

Table 1. 4 hr treatment

Upregulated	Downregulated
lymphocyte specific 1	Cell division cycle 25 homolog
orosomucoid 1	expressed sequence AU021460
chloride channel 3	expressed sequence AW539821
DNA segment, Chr 2, ERATO Doi 120, expressed	expressed sequence C78101
SoxLZ/Sox6 leucine zipper binding protein in testis	polo-like kinase homolog, (Drosophila)
	Rab6, kinesin-like
	RIKEN cDNA A030007L17 gene
	Ttk protein kinase

Table 2. 36 hr treatment

Upregulated	Downregulated
expressed sequence AI507170	expressed sequence AI467481
expressed sequence AU022351	frizzled homolog 1, (Drosophila)
expressed sequence AW146114	interferon activated gene 204
histocompatibility 2, D region locus 1	oxysterol binding protein-like 1A
lymphocyte antigen 75	procollagen, type XI, alpha 1
protein kinase inhibitor, alpha	ribonucleotide reductase M2
reticulon 2 (Z-band associated protein)	
RIKEN cDNA 1200008D14 gene	
RIKEN cDNA 5830443C21 gene	
serum amyloid A 3	
tumor protein D52-like 1	
ubiquitin specific protease 14	
vesicle transport through interaction with t-SNAREs 1 homolog	

Table 3. 48 hr treatment

Upregulated	Downregulated
expressed sequence AU02235	hyaluronan synthase 2
histocompatibility 2, D region locus 1	lumican
hyaluronan synthase 2	Ras-related associated with diabetes
IMAGE:4236882, mRNA, complete cds	WNT1 inducible signaling pathway protein 2
myeloid leukemia factor 1	
lipocalin 2	expressed sequence AI747191
lymphocyte antigen 75	cytochrome P450, 7b1
metallothionein 2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2
Mus musculus, clone MGC:19199	cysteine knot superfamily 1, BMP antagonist 1
orosomucoid 1	chemokine orphan receptor 1
protein kinase inhibitor, alpha	prostaglandin I2 (prostacyclin) synthase
protein tyrosine phosphatase, non-receptor type 16	lymphocyte specific 1
reticulon 2 (Z-band associated protein)	tumor necrosis factor, alpha-induced protein 2
RIKEN cDNA 1300010A20 gene	expressed sequence AW550625
RIKEN cDNA 2310032D16 gene	fibroblast growth factor 7
RIKEN cDNA A030007L17 gene	interleukin 1 receptor-like 1
serine (or cysteine) proteinase inhibitor, clade I (neuroserpin), member 1	latent transforming growth factor beta binding protein 1
serum amyloid A 3	tumor protein D52-like 1
tumor protein D52-like 1	ubiquitin specific protease 14
vesicle transport through interaction with t-SNAREs 1 homolog	vascular cell adhesion molecule 1
	solute carrier family 8 (sodium/calcium exchanger), member 1
	small inducible cytokine B subfamily, member 5
	runt related transcription factor 2
	RIKEN cDNA 0610007L05 gene

Table 4. TSA treatment

Upregulated	Downregulated
serum amyloid A 3	annexin A8
ubiquitin specific protease 14	cell division cycle 25 homolog C (S. cerevisiae)
aldehyde dehydrogenase family 1, subfamily A4	expressed sequence AI528536
RIKEN cDNA 1200008D14 gene	keratin complex 1, acidic, gene 13
protein kinase inhibitor, alpha	polo-like kinase homolog, (Drosophila)
expressed sequence AW212668	
lymphocyte antigen 75	

CONTRIBUTION OF COLLABORATORS

The author is responsible for much of the data analysis and experimental work covered in this thesis. Notable exceptions include the construction of the pGEX2T-C/EBPβ construct by Ms. Louise Pope. The mutation of lysines 98, 101 and 102 of this same construct was completed by Ms. DongMei Wu. Ms. Wu also provided technical assistance for thecompletion of the chromatin immunoprecipitations described in this work as well as for the Northern analysis of HDAC1 mRNA levels. The author was responsible for the planning of experiment, the cell culture and the data analysis.

Microarray data mentionned briefly in the discussion were performed at the Montreal Genome Centre at McGill University by our collaborator Dr. Rob Sladek. The author provided the RNA samples for study, and was responsible for classifying and analysing the resulting data.