REGULATION OF THE ACETYLATION OF C/EBPβ AND ITS FUNCTIONAL CONSEQUENCES

by

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To my husband, Fred Rivera,
and my familia

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CHAPTER I

INTRODUCTION

Because of the increasing incidence of obesity worldwide, the molecular mechanisms controlling adipogenesis have become a critical area of research (1). Studies related to adipogenesis have led to the identification of genes involved sequentially in the differentiation of fat cells, such as C/EBPβ, C/EBPδ, C/EBPα, and PPARγ (2, 3). This thesis focuses on the regulation of the activity of CCAAT/Enhancer Binding Protein (C/EBP) β, an early inducer of adipogenesis, which mediates the transcriptional activation of C/EBPα and PPARγ. Because C/EBPβ associates with the nuclear coactivator p300, which contains acetyltransferase activity (4), we hypothesized that C/EBPβ is acetylated and that acetylation of C/EBPβ may contribute to its role in adipogenesis. This thesis investigates a model in which dynamic regulation of the acetylation of C/EBPβ contributes to its ability to mediate transcriptional activation of target genes associated with adipogenesis. Our findings suggest that acetylation of C/EBPβ at lysine 39 contributes to adipogenesis and adipocyte function, thereby adding to our understanding of cellular mechanisms relating to obesity, and revealing potential therapeutic approaches for obesity and related health problems.

Adipocytes and Adipogenesis

Adipocytes, or fat cells, are derived from mesenchymal precursors and expand extensively after birth (2). Collectively, fat is a tissue that has long been known to serve as an energy depot by storing triglycerides. Recent observations demonstrate that adipose tissue is not just a passive storage organ. Adipose tissue has the capacity not only for lipid transport and synthesis, but also for immune function and secretion of hormones such as leptin, which regulates appetite and metabolism, adiponectin, which increases sensitivity of target cells to insulin, and resistin, which increases resistance to insulin (1). Cell culture models have been developed to study the molecular basis of adipogenesis, or adipose cell differentiation. Two widely used cell lines are 3T3-L1 and 3T3-F442A preadipocytes, which were derived from mouse embryo fibroblasts that accumulated triacylglycerol lipid droplets (5, 6).

A variety of inducers can initiate the process of adipogenesis in confluent monolayers of preadipocyte fibroblasts. The inducers include agents which increase cAMP levels, glucocorticoids and IGF-1. In cultured cell models, an adipogenic "cocktail" that includes isobutylmethylxanthine (MIX), dexamethasone, and insulin, in the presence of fetal bovine serum (FBS), referred to here as "MDI Medium", is often used to induce adipose conversion (3, 7). MIX is a cAMP phosphodiesterase inhibitor which increases cellular cAMP (8, 9). It was originally postulated that MIX is capable of inducing CCAAT/Enhancer Binding Protein (C/EBP) β expression, while dexamethasone activates C/EBPδ (8); the two C/EBP family members are important early inducers of adipogenesis (discussed in detail below) (3, 10). It has also been reported that

dexamethasone, a potent synthetic glucocorticoid (3), increases C/EBPβ expression and potentiates transcriptional activation by C/EBPβ (11). Since the C/EBPβ promoter contains C/EBP binding sites, the increase attributed to C/EBPβ may be at least partially due to indirect activation of C/EBPβ by C/EBPδ. Insulin also increases C/EBPβ and C/EBPδ expression (12). Fetal bovine serum likely provides other factors that favor adipogenesis, including growth hormone (GH). GH has been found to be a necessary component in the adipose conversion of 3T3-F442A cells (13); when GH was immuno-depleted from FBS, differentiation could not be induced in these cells (14).

Transcription Factors Involved in Adipose Differentiation

Transcriptional activation involves the regulation of genes by transcription factors that bind to the regulatory sequences of target genes. Transcription via transcription factors is regulated on several levels, including: the binding of transcription factors to target sites, post-translational modifications of transcription factors themselves, binding of coactivators and corepressors, and the location and post-translational modification state of histones (15). This thesis will focus on one post-translational modification, acetylation, of the transcription factor C/EBPβ.

Adipose differentiation involves the sequential activation of transcription factors. Two of these transcription factors, C/EBPβ and C/EBPδ, are induced early in preadipocyte conversion to adipocytes. C/EBPβ and C/EBPδ initiate a cascade of transcription factors that includes C/EBPα and Peroxisome Proliferator-Activated Protein (PPAR) γ (16), two master regulators of adipogenesis (3). Although these transcription

factors have functions outside of adipose differentiation, this thesis will focus on their roles in adipogenesis.

PPARγ2

PPARγ2, a nuclear hormone receptor that heterodimerizes with Retinoic acid-X-Receptor (RXR) α, binds and activates genes involved in adipogenesis, such as adipocyte Protein 2 (aP2/) and PhosphoEnolPyruvate CarboxyKinase (PEPCK) (17, 18). aP2 is a cytosolic fatty acid binding protein that is induced by fatty acids, and PEPCK is an enzyme involved in gluconeogenesis (3). PPARγ2 is a splice variant of PPARγ1 that includes a different 5' untranslated sequence in the gene and 30 more amino acids on its N-terminus (17). The inducible inactivation of the PPARγ gene in mouse adipose tissue leads to progressive lipodystrophy in fat tissue, supporting a role for PPARγ in adipocyte maintenance (19). PPARγ2 is expressed exclusively in adipose tissue, and ectopic PPARγ2 expression in NIH-3T3 and Swiss-3T3 cells promotes their differentiation to adipocytes (18, 20).

C/ EBPα

C/EBPα, the founding member of the C/EBP family of transcription factors, is expressed in liver, intestine and lung, as well as adipose tissue (3). C/EBPα is involved in termination of mitotic clonal expansion, when cells synchronously reenter the cell cycle and undergo several rounds of mitosis, during adipose differentiation. C/EBPα also mediates activation of adipocyte genes that encode aP2, glucose transporter (GLUT) 4, and Stearoyl-CoA Desaturase (SCD) 1, as well as autoactivation of C/EBPα (3). GLUT4

encodes an insulin-responsive glucose transporter (21, 22), and SCD1 is involved in lipogenesis and the desaturation of saturated fatty acids (23). Expression of antisense C/EBPα RNA in cells blocked expression of these genes, and also blocked accumulation of cytoplasmic triacylglycerol. Rescue could be induced by expressing a complementary sense RNA (24). When C/EBPα is overexpressed, it induces adipose differentiation, even in the absence of differentiation medium (25, 26). C/EBPα knockout mice die shortly after birth due to severe hypoglycemia, which is most likely a consequence of reduced glycogen synthesis in the liver. Moreover, hepatocytes and adipocytes also fail to accumulate lipids (27). Interestingly, C/EBPα does not promote adipogenesis in PPARγ-deficient cells, but PPARγ does promote adipogenesis in C/EBPα-deficient cells, suggesting that, even though there is cross-regulation, PPARγ is both necessary and sufficient for adipogenesis (28-30). It is interesting to note that C/EBPα -/- cells that have been induced to differentiate with PPARγ are extensively insulin resistant (28). This indicates that, although C/EBPα may not be necessary for adipose conversion in cell culture, it is still necessary for other functions such as insulin sensitivity.

C/EBPδ

C/EBPδ is expressed in adipose tissue, lung and intestine (29, 31, 32), as well as in osteoblasts (33), mammary cells (34), and hepatocytes (35, 36). C/EBPδ (-/-) mice have normal white adipose tissue, but have reduced lipids in their brown adipose tissue, as well as reduced expression of Uncoupling Protein-1 (UPC1), important in thermoregulation (37). Although C/EBPδ plays a role in adipose differentiation, C/EBPβ is reported to play a more prominent role, especially in the activation of PPARγ (38).

Inhibitory transcription factors

Several key transcription factors can inhibit adipose differentiation. On the C/EBPα promoter, it is thought that the C/EBP binding site is unavailable as a docking site until C/EBP Undifferentiated Protein (CUP) dislodges from the nearby CUP regulatory element (39). C/EBP homologous Protein (CHOP), or C/EBPζ, heterodimerizes with C/EBP family members, but because it contains an altered DNA binding region, it can act as a dominant negative protein in adipocyte differentiation by interfering with C/EBPβ function (40, 41). GATA2, which normally promotes haematopoiesis and urogenital development, binds to C/EBPs. Forced expression of GATA2 inhibits the ability of C/EBPs to transactivate PPARγ, thus inhibiting adipogenesis (42). The nuclear protein Eight Twenty-One (ETO), whose expression decreases during adipose differentiation, can inhibit C/EBPβ function through a direct interaction with C/EBPβ (43).

C/EBPβ is an Early Inducer of Adipogenesis

C/EBPβ is a transcription factor that is present in cells in three forms: Full length Liver-enriched Activating Protein 1 (LAP1, residues 1-296) (44), LAP2 (residues 22-296), and Liver Inhibitory Protein (LIP, residues 151-296). LIP is a shorter form that lacks the N-terminal transactivation sequence and is inhibitory (45, 46).

The N-terminal transcriptional activation domain of C/EBPβ, specifically amino acids 85-95, is thought to interact with the basal transcriptional machinery (47). The N-terminus is also known to interact with important coactivators, including p300 (4) and

CREB-Binding Protein (CBP) (48). Further, unspecified residues in the N-terminal transcriptional activation domain interact with Silencing Mediator for Retinoid and Thyroid hormone receptors (SMRT), a corepressor complex with deacetylase properties (49), and residues 1-22 in C/EBPβ interact with the Swi/Snf complex, an ATP-dependent helicase complex responsible for chromatin remodeling (50). The N-terminus is critical for transcriptional activation (51), perhaps because of its ability to interact with key coactivators or corepressors.

As a basic-leucine zipper (B-Zip) protein, C/EBPβ also contains a C-terminal basic DNA-binding domain and a leucine zipper which mediates dimerization (3) (Fig. 1.1). The DNA-binding domain binds to the DNA sequence ATTGCGCAAT, or a variation of this consensus C/EBP sequence (31). The leucine zipper is composed of five heptad repeats that assemble into a dimeric coiled-coiled structure. This interaction is responsible for homodimerization as well as heterodimerization with other B-Zip transcription factors, particularly with other members of the C/EBP family. The dimer interacts with DNA via the basic region, forming a “scissors-grip” arrangement (52). Dimerization is a prerequisite for binding of C/EBPβ to DNA (29, 52, 53). Recent reports also indicate that phosphorylation of C/EBPβ (at T179/T188 or S184/T188) leads to a three-dimensional change in C/EBPβ that can regulate its dimerization (54). When phosphorylation increases at these sites, both glutaraldehyde-induced crosslinking of dimers, as well as binding to DNA are also increased. C/EBPβ also contains negative regulatory domains that are thought to mask its transcriptional activation domain (51, 55). It is postulated that inactive C/EBPβ is folded in such a way that the N- and C-termini are in close proximity. This folding inhibits C/EBPβ activity by making the transcriptional

Fig. 1.1 mouse C/EBPβ

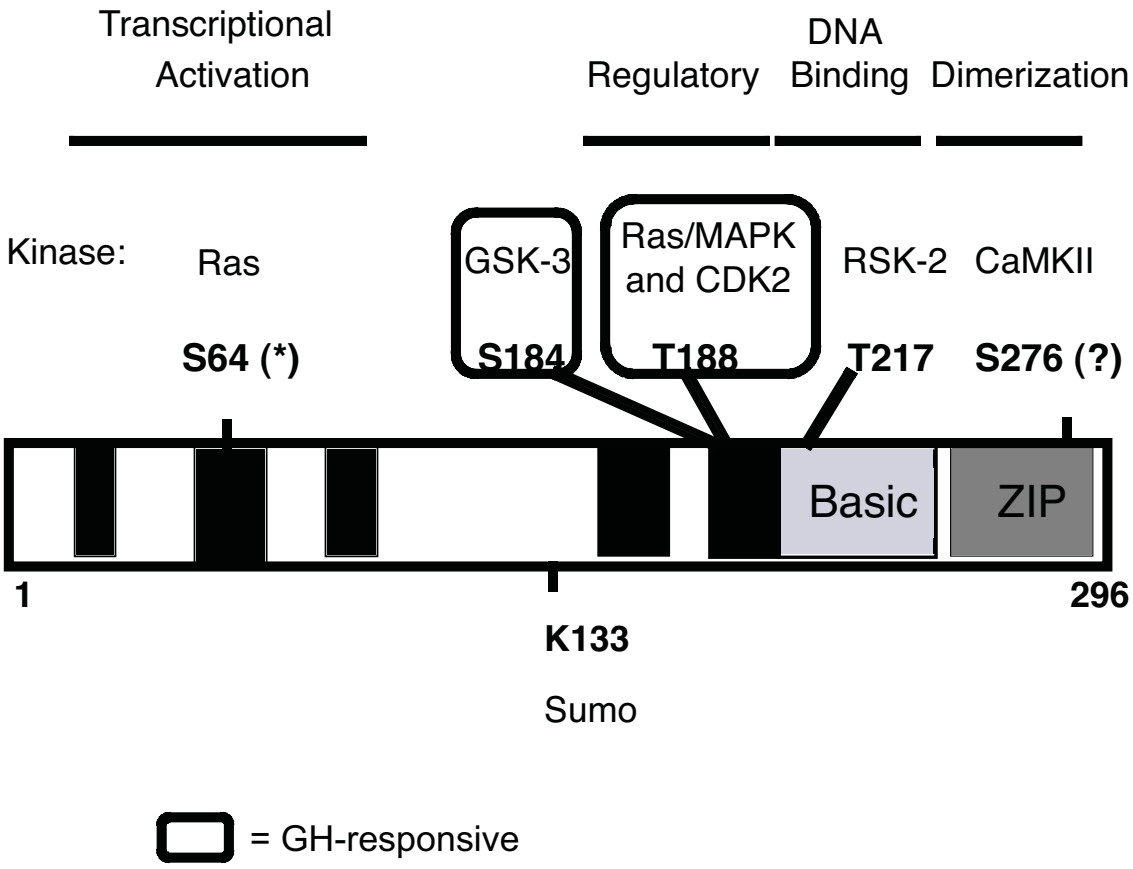

Fig 1.1. Domains and phosphorylation sites within C/EBPβ. Diagram indicates the different domains of C/EBPβ. Also indicated are phosphorylation sites within mouse C/EBPβ, and their respective kinases, as well as a SUMO site. (*) =indicate phosphorylation sites have been found to be phosphorylated in rat, and that are conserved in mouse.

activation domain inaccessible to regulatory elements because of its binding to negative regulatory domains. When C/EBPβ is phosphorylated, it modulates the three-dimensional structure of C/EBPβ molecule so that the transcriptional activation domain is available for interaction with regulatory proteins.

The role of C/EBPβ in adipose differentiation

C/EBPβ is expressed in adipose, hepatic, and a variety of other tissues (31, 46, 56-60). In hepatocytes, C/EBPβ promotes proliferation in response to Transforming Growth Factor (TGF) α (61, 62); hepatocytes in C/EBPβ (-/-) mice exhibit a reduced regenerative response (63). C/EBPβ (-/-) mice also exhibit defects in the gluconeogenic pathway attributed to reduced PEPCK levels (64). Keratinocytes from C/EBPβ (-/-) mice are resistant to carcinogen-induced skin tumor development (60), indicating a role for C/EBPβ in promoting their proliferation. C/EBPβ (-/-) mice also have an increased susceptibility to infection, which is thought to be due to impairments in bacteria killing and cytotoxicity, although the mechanism is obscure (65). Further, C/EBPβ plays an important role in the hematopoietic system (66) and female reproduction (67). Although C/EBPβ plays many roles in a variety of cellular functions, this thesis will focus on the role of C/EBPβ in adipogenesis.

C/EBPβ, which induces expression of C/EBPα and PPARγ, is an early component of the transcriptional cascade of adipogenesis (3). In C/EBPβ (-/-) C/EBPδ (-/-) mice, a significant decrease in adipose tissue mass was observed, despite normal expression of C/EBPα and PPARγ (37). Cells from C/EBPβ (-/-) mice exhibited a significant reduction in adipose differentiation (37), supporting a prominent role for

C/EBPβ in adipose differentiation, especially in the activation of PPARγ (38). C/EBPβ (-/-) neonates show no other gross physical changes. However, about 35% die by about 24 hrs after birth and females are infertile. C/EBPβ (-/-) mice are also refractory to diet-induced obesity, reflected in lower levels of blood triglycerides, free fatty acids, cholesterol, and hepatic triglyceride accumulation (68).

Early studies indicate that overexpression of C/EBPβ in NIH-3T3 cells is not sufficient to induce adipogenesis, whereas expression of C/EBPα is sufficient (26). Ectopic expression of C/EBPβ in NIH-3T3 cells, along with MDI medium, can induce adipose differentiation (8, 38). Further, adenoviral expression of C/EBPβ in C/EBPβ(-/-) murine embryonic fibroblasts (MEFs), coupled with the addition of MDI medium, promoted expression of adipocyte markers in these cells (69). In 3T3-L1 cells, Yeh *et al.* observed that overexpression of C/EBPβ led to adipocyte differentiation, even in the absence of hormonal stimulation (8).

After addition of MDI medium, preadipocytes undergo several rounds of mitotic clonal expansion, which are thought to be necessary for differentiation to progress (69), although this is controversial (70). Tang *et al.* (69) found that, following MDI induction of differentiation of 3T3-L1 cells, mitosis is induced, based on activation of cdk2/cyclinE/A, turnover of p27, hyperphosphorylation of Rb, and incorporation of radiolabeled thymidine into DNA. Further, when cdk was inhibited with roscovitine, not only mitotsis, but also adipogenesis was blocked. During mitotic clonal expansion, C/EBPβ undergoes phosphorylation changes necessary for its ability to bind DNA (see below) (69, 71). After a lag period (~10hrs), C/EBPβ acquires the ability to bind to the promoters of C/EBPα and PPARγ genes. Since mitotic clonal expansion is necessary for

differentiation, the lag time in the expression of C/EBPα and PPARγ is thought to be necessary because C/EBPα and PPARγ are both antimitotic (72, 73).

Posttranslational Modifications Modulate Transcriptional Activation by C/EBPβ

Phosphorylation of C/EBPβ is one mechanism to regulate its function. Phosphorylation occurs at multiple sites on C/EBPβ, and is mediated by different kinases (Fig 1.1). In NIH-3T3 cells, expressed human C/EBPβ is phosphorylated at T235 (which corresponds to T188 in mouse) in the presence of Ras, and increases transcription of the Interleukin-6 (IL-6) promoter, a C/EBPβ target gene (74). Lipopolysaccharide (LPS) increases Ras-induced phosphorylation of rat C/EBPβ at S64 (conserved in mouse). This modification increases both IL-6 mRNA and protein in murine B lymphoblasts (75). Phosphorylation of rat C/EBPβ at S276 (conserved in mouse) mediated by Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) increases transactivation of a promoter that contains sites for C/EBPβ binding sites in pituitary cells (76). Phosphorylation of rat C/EBPβ at S105 or of mouse C/EBPβ at T217 by p90 ribosomal S6 kinase stimulates proliferation in differentiated hepatocytes treated with TGFα (62).

Phosphorylation of C/EBPβ can also be inhibitory. Protein Kinase C-mediated phosphorylation at S240 of rat C/EBPβ is reported to attenuate DNA binding in hepatocytes (77). PKC did not directly phosphorylate C/EBPβ *in vitro,* so it most likely induces a downstream kinase to directly phosphorylate C/EBPβ. In resting osteoblasts, Glycogen Synthase Kinase (GSK)-3β mediates constitutive phosphorylation of rat

C/EBPβ at T189, S185, S181, and S177, which is inhibitory to C/EBPβ binding to DNA (78).

C/EBPβ is found exclusively in the nucleus of 3T3-L1 preadipocytes and adipocytes (71) and in 3T3-F442A preadipocytes (79). In 3T3-F442A preadipocytes, C/EBPβ phosphorylated at T188 is induced to translocate to heterochromatin in response to GH (79). In other cell lines, C/EBPβ can shuttle between the cytoplasm and the nucleus. In PC12 cells, C/EBPβ was reported to translocate to the nucleus in a cAMP/PKA-dependent manner (80). In DKO-1 colorectal cancer cells, the antioxidant pyrrolidinedithiocarbamate (PDTC) induces phosphorylation of C/EBPβ at S299, which is suggested to mediate translocation to the nucleus in a PKA-dependent manner (81). In mouse hepatocytes, Tumor Necrosis Factor (TNF) α induces C/EBPβ phosphorylation at S239, which then facilitates CRM1-mediated nuclear export (82). S239 lies in "motif A" (residues 226-246), which has been reported to function as a nuclear localization signal (83).

C/EBPβ contributes to GH-mediated activation of c-*fos*

The adipogenic conversion of 3T3-F442A preadipocytes is dependent on growth hormone (GH) (14). C/EBPβ protein levels increase in response to GH in 3T3-F442A preadipocytes (84). C/EBPβ is also a critical mediator of the activation by GH of c-*fos*, a proto-oncogene target of C/EBPβ (85-88). When C/EBPβ is decreased by siRNA directed against C/EBPβ in either 3T3-F442A preadipocytes or Chinese Hamster Ovary (CHO) cells, GH can no longer mediate activation of c-*fos* (89). Thus, C/EBPβ contributes to GH-mediated activation of c-*fos*.

The importance of the phosphorylation of C/EBPβ in GH-stimulated transcription is suggested by reports of multiple changes in the phosphorylation state of C/EBPβ in response to GH (Fig. 1.1). Isoelectric focusing identified at least six distinct phosphorylated forms of C/EBPβ that are regulated by GH in 3T3-F442A cells (90). A Mitogen-Activated Protein Kinase (MAPK) substrate site at T235 in human C/EBPβ (74), which corresponds to T188 in murine C/EBPβ, is rapidly and transiently phosphorylated in response to GH in an Extracellular signal Regulated Kinase (ERK) 1/2-dependent manner; such phosphorylation alters its ability to activate transcription of target genes such as c-*fos* (90). Mutation at that MAPK phosphorylation site of C/EBPβ almost completely abrogates the stimulation of the c-*fos* promoter in response to GH, indicating that phosphorylation at the MAPK substrate site is required for GH to activate c-*fos*.

It is notable that while phosphorylation of C/EBPβ at T188 is rapid and transient, dephosphorylation of C/EBPβ at aGSK-3 substrate site (91) may occur only after 60 minutes of GH treatment. The delayed dephosphorylation may mediate attenuation of c-*fos* transcription; it is dependent on activation by GH of phosphatidylinositol 3-Kinase (PI3K) and downstream AKT, which leads to inhibition of GSK-3 activity. Thus, regulation by GH of the phosphorylation state of C/EBPβ, mediated by MAPK and PI3K-AKT signaling cascades, is an important component of the mechanisms of GH-stimulated transcription of c-*fos*.

SUMOylation of C/EBPβ may also contribute to GH-mediated activation of c-*fos*. SUMOylation is a posttranslational modification which is often associated with negative regulation of transcription (92, 93). SUMOylation involves the conjugation of members

of the Small Ubiquitin-Like MOdifier (SUMO) family to acceptor lysine residues in target proteins (94). Lysine 133 in murine C/EBPβ is SUMOylated (95-97). Previous data indicate that mutation of C/EBPβ at K133 elevates basal c-*fos* transcription to a level where it cannot be further stimulated by GH (97), opening the possibility that deSUMOylation, and consequent relief of an inhibitory effect of SUMO, may contribute to the ability of C/EBPβ to activate transcription in response to GH.

Phosphorylation of C/EBPβ and adipogenesis

Phosphorylation of C/EBPβ at the MAPK/ERK substrate site T188 increases during adipogenesis in 3T3-L1 cells (98, 99). Phosphorylation at this site is detected within 4 hours after adipose differentiation commences in response to MDI medium, which is when MAPK is present and active (phosphorylated) within the nucleus of 3T3-L1 cells (99). It has also recently been found that although T188 is initially phosphorylated by MAPK, phosphorylation at this site is maintained by cdk2 (100). It is not until ~10 hrs after differentiation commences that C/EBPβ is phosphorylated at the GSK-3 sites T179 and S184, concurrent with the timing of translocation of GSK3 to the nucleus. This GSK3-mediated phosphorylation of C/EBPβ, which is dependent on prior phosphorylation at T188, then promotes maximal DNA-binding by C/EBPβ on promoters of adipocyte genes such as C/EBPα and aP2 (99). In agreement with these studies, Park *et al.* observed that phosphorylation of C/EBPβ at T188 is required to induce expression of C/EBPα and adiponectin (98). Thus, phosphorylation of C/EBPβ appears to contribute to the role of C/EBPβ in adipogenesis.

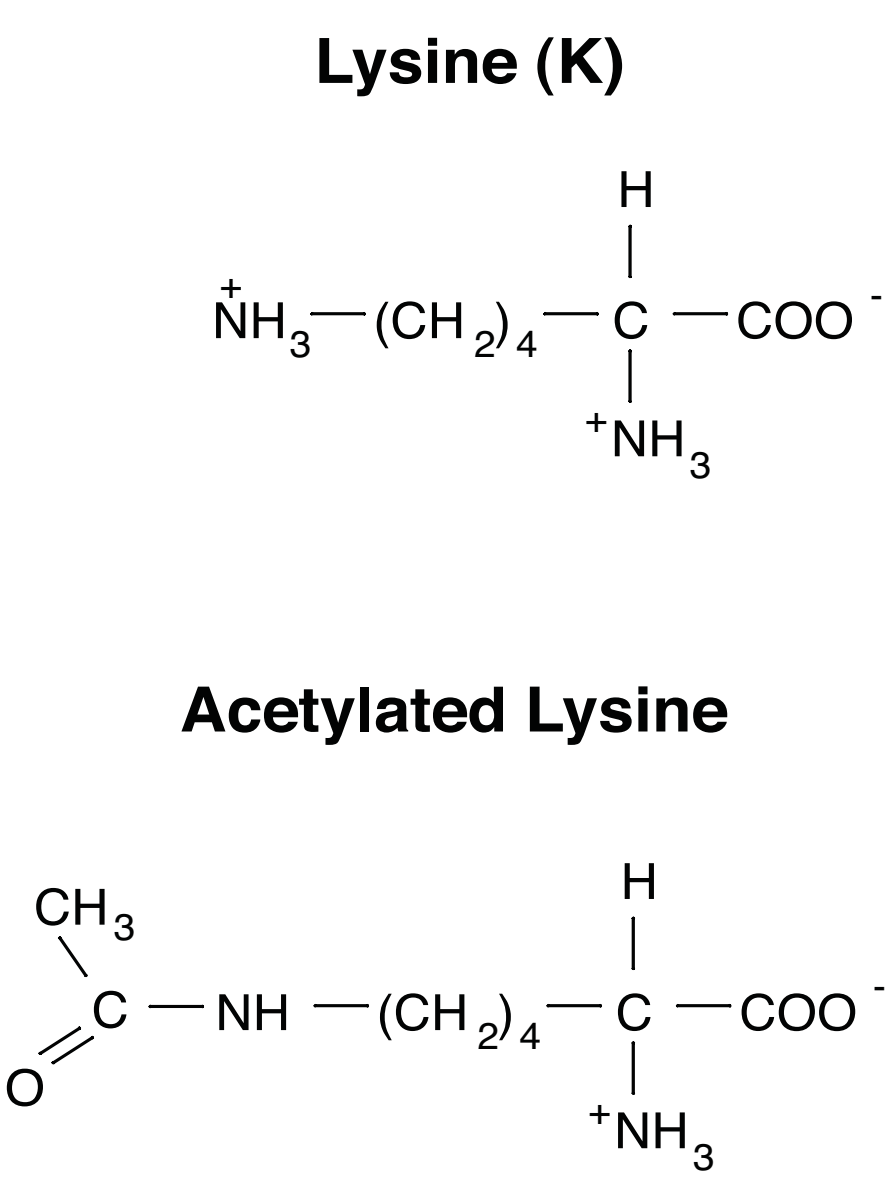

Fig 1.2. Acetylation of a lysine residue. Shown are both a nonacetylated and acetylaed lysine residue. Adapted from Li *et al.* (104).

Acetylation of Nuclear Proteins

In addition to phosphorylation and sumoylation, a growing list of modifications, including acetylation, methylation, and ubiquitination, regulate the transcriptional regulatory potential of multiple nuclear proteins (101). In the case of histone acetylation, acetyltransferases add an acetyl group to the ε-amino group of multiple lysine side chains in the N-terminal tail region (Fig 1.2). Acetylation is associated with chromatin remodeling which makes DNA more accessible to the transcriptional regulatory machinery (102). Histone methylation on arginines is usually involved in gene activation, while histone methylation on lysines is usually repressive (101). Histone ubiquitination on lysines may also be involved in modulating gene activity. Combinations of such modifications, now considered to serve as a "histone code," further modulate chromatin structure (103).

The acetylation state of nuclear proteins is highly susceptible to regulation by histone acetyltransferases (HATs) and/or histone deacetylases (HDACs). Although the original terminology referred to these as "histone" enzymes, they are now recognized to modulate the addition of acetyl groups to lysines of a wide array of non-histone substrates as well. Unlike phosphorylating kinases, there is no reported evidence of "acetylation cascades", or the consecutive procession of acetylase activity, to modify a biological signal (105).

Acetyltransferases

Several families of protein acetylasetransferases are exemplified by CBP/p300, P300/CBP Associated Factor / General Control Nonderepressible-5 (PCAF/GCN5), TATA box binding protein Associated Factor (TAF) II 250 (TAFII250), Steroid Receptor Coactivator (SRC) 1, and MOZ (Monocytic leukemia Zinc finger protein (105). Two well-studied examples of acetyltransferases are CBP and the highly homologous p300 (106, 107). CBP and p300 were initially identified through their respective associations with transcription factor CREB and the viral protein E1A (108-110). In humans, Rubenstein-Taybi Syndrome is associated with CBP heterozygosity (111-113). Rubenstein-Taybi Syndrome is a developmental disorder characterized by mental retardation, unusual facial appearance and broad digits. p300 heterozygosity has also been reported in tumor tissue from several carcinoma patients (111, 113). In mice, CBP or p300 homozygosity is embryonic lethal (112, 114). Because of the diversity of defects and the many binding partners of these coactivators, it has been difficult to pinpoint any one reason for the lethality.

In cultured cells, CBP and p300 are localized only in the nucleus (110, 112). However, in tissue, CBP and p300 can be found in both the nucleus and the cytoplasm. For example, in murine notochord cells, p300 translocates from the cytoplasm to the nucleus during embryonic development (112). In primordial ovarian follicles, CBP and p300 are initially found in the cytoplasm of oocytes, but p300 and CBP move to the nucleus during later growth stages (115). Cytoplasmic localization is not surprising considering that these acetyltransferases have been found to acetylate both cytoplasmic and nuclear substrates.

Interestingly, during adipose conversion of 3T3-L1 and 3T3-F442A cells, p300 levels increase (116) and p300/CBP knockdown suppresses differentiation of 3T3-L1 cells (117). Further, p300 has been found to enhance C/EBPα- (118) and PPARγ-mediated (117, 119) transcription of adipogenic target genes. These data indicate that p300 contributes to adipose differentiation.

Deacetylases

In contrast to acetylases, HDACs catalyze removal of acetyl groups from substrates. HDACs are divided into three classes (120, 121): Class I HDACs, which include HDACs 1, 2, 3, 8, and most likely 11, resemble yeast deacetylase RPD3 in terms of sequence similarity, and are localized primarily in the nucleus. HDAC1 and HDAC2 are part of high-molecular-weight corepressor complexes, Sin3 and NuRD-Mi-2. HDAC3 is a component of N-CoR and SMRT corepressor complexes. Class II HDACs, 4, 7, 9, and 10, resemble yeast deacetylase HDA1, and exhibit tissue-specific expression. Class III HDACs are NAD^+-dependent, and are the least characterized of the HDACs. There are several classes of HDAC inhibitors. Trichostatin A (TSA) inhibits class I and II HDACs by directly binding to their catalytic domain, and nicotinamide inhibits class III HDACs by promoting a base-exchange reaction with NAD^+ so that it can no longer act as a cofactor in HDAC III-mediated deacetylation (122). Use of HDAC inhibitors has become an emerging approach to treating proliferative disorders such as cancers (120, 123).

During adipose conversion of 3T3-L1 and 3T3-F442A cells, HDAC levels decrease (116). Further, HDAC1 overexpression decreases adipocyte conversion in 3T3-

L1 cells (116, 124). Recently, knockdown by siRNA of Sirt2, a class III HDAC, was reported to promote 3T3-L1 adipose differentiation, while overexpression of Sirt2 impaired it (125). Sirt2, which is cytoplasmic, increases acetylation of Foxo1, which in turn increases Foxo1 phosphorylation, and excludes it from the nucleus. This is postulated to contribute to the observed increase in adipose differentiation since Foxo1 is known to bind and repress the PPARγ promoter (126). These studies suggest that overall HDAC levels decrease during adipose conversion, which is consistent with the increase observed in overall acetyltransferase levels.

As reported in the literature, the consequences of inhibiting HDACs during adipose differentiation have been inconsistent. Lagace and Nachtigal (127) reported that when 3T3-L1 cells are treated with TSA (1-10nM) or valproic acid (1mM, an HDAC inhibitor in the same family as sodium butyrate), there is an impairment in adipose differentiation, as well as a decrease in the expression of PPARγ and C/EBPα. This decrease in differentiation was not surprising in light of data that mitotic clonal expansion is an early step in adipose differentiation (128), and that TSA blocks both cell cycle progression in HeLa cells (129) and proliferation in hepatocellular carcinoma cells (130).

In contrast, others report an increase in adipose differentiation with HDAC inhibition (116, 124, 131). TSA (400nM) or valproic acid (10mM) increased adipose differentiation in 3T3-L1 cells, but only in the absence of dexamethasone; no enhancement was observed for adipose differentiation in the presence of dexamethasone (124). With 1.5mM valproic acid treatment, Fajas *et al.* (131) demonstrated an increase in adipocyte differentiation in 3T3-L1 cells. Yoo *et al.* (116) observed an increase in 3T3-L1 differentiation with sodium butyrate (500μM). These studies suggest that

acetylation-mediated chromatin remodeling is necessary for induction of important genes for adipose differentiation. Thus, the mechanism of how HDACs, and associated changes in acetylation of their substrates, regulate adipose differentiation is unclear at present.

Functional Consequences of Acetylation of Transcription Factors

Acetylation of nuclear proteins was first detected in histones and is viewed as a part of a mechanism allowing DNA to become accessible to transcription regulatory machinery (132, 133). It is now recognized that many cellular proteins are regulated by acetylation. In the nucleus, acetylation of a growing number of transcription factors is reported to have broad impact on their function. For example, the acetylation of the tumor suppressor p53 stabilizes it by preventing its ubiquitination by Mdm2, thereby allowing p53 to enter the nucleus to activate target genes (104). Acetylation of p53 at lysines 320, 373, and 382 increases its binding to cognate DNA (134, 135). Acetylation is also reported to increase nuclear localization of, and thus transcription activation by, NF-κB, by weakening its interaction with IκBα, which sequesters NF-κB in the cytoplasm (136). Acetylation of GATA-1 was found to increase its binding to DNA, thereby stimulating GATA-1 dependent transcription (137).

Other functional consequences of acetylation include promoting interaction of the nuclear import factor importin-α with importin-β (138). Ku70 is unable to bind and sequester pro-apoptotic BAX when Ku70 is acetylated (139). Acetylation in the DNA-binding domain of HMGI(Y), a complex required for enhancesome assembly, is

inhibitory, decreasing its DNA-binding ability and weakening its transcriptional potency (105, 140). Acetylation has also been observed to increase the stability of E2F1. P/CAF, but not P/CAF with the HAT domain mutated, acetylates and concomitantly increases the stability of E2F1 by an unknown mechanism (141). Thus, acetylation can modify the function of a variety of cellular proteins by altering binding to DNA, binding to other proteins, and regulating degradation.

Aims of Thesis

Control of transcription by transcription factors involved in adipose differentiation and potentially obesity, is an area of study of increasing interest as the prevalence of obesity rises. One mode to control transcription factors is through posttranslational modifications such as acetylation, which we now recognize as a powerful determinant of transcription factor action. This thesis explores the acetylation of C/EBPβ and its functional consequences, particularly in the regulation of target genes involved in adipogenesis.

Chapter II presents studies conducted to demonstrate the acetylation of C/EBPβ by p300 and P/CAF. Mapping studies were carried out to pinpoint acetylated amino acids within the C/EBPβ sequence. These studies provide the foundation for further studies as to the functional consequences of acetylation of C/EBPβ at these lysines. Since C/EBPβ is a transcription factor, whether acetylation of C/EBPβ alters transcriptional activation of the target gene c-*fos* was examined. c-*fos* has proven to be an excellent model gene not only of C/EBPβ transcriptional potential, but also of GH-

mediated gene activation. Whether acetylation and phosphorylation of C/EBPβ work in coordination was also investigated.

C/EBPβ is also an early inducer of adipogenesis (10). It induces the expression of the master regulators of adipogenesis, C/EBPα and PPARγ (3). Chapter III explores whether endogenous C/EBPβ is acetylated in adipocytes, and what roles acetylation of C/EBPβ may play during adipogenesis, particularly in the activation of key regulators of adipogenesis. Further, the deacetylation of C/EBPβ by HDACs was examined to characterize the dynamic regulation of the acetylation of C/EBPβ.

Acetylation of C/EBPβ is likely to be one component of a complicated puzzle that contributes to adipogenesis. Regulation of adipose differentiation involves control of many genes to convert preadipocytes to adipocytes. Multiple posttranslational modifications of regulatory proteins most certainly contribute to this control. Ultimately, targeting these posttranslational modifications may lead to treatment options to overcome problems related to obesity and obesity-related disorders.

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