CHAPTER II

C/EBPβ IS ACETYLATED AT MULTIPLE LYSINES: ACETYLATION OF C/EBPβ AT LYSINE 39 MODULATES ITS ABILITY TO ACTIVATE TRANSCRIPTION

Abstract

Transcription factor function can be modulated by post-translational modifications. Because the transcription factor C/EBPβ associates with the nuclear co-activator p300, which contains acetyltransferase activity, acetylation of C/EBPβ was examined to understand its regulation and function. C/EBPβ is acetylated by acetyltransferases p300 and p300/CBP Associated Factor (P/CAF). Endogenous C/EBPβ in 3T3-F442A preadipocytes is also recognized by an acetyl-lysine specific antibody. Analysis of truncations of C/EBPβ and peptides based on C/EBPβ sequences identified multiple lysines within C/EBPβ that can be acetylated. Among these, a novel acetylation site at lysine (K) 39 of C/EBPβ was identified. Mutation of K39 to arginine or alanine impairs its acetylation and the ability of C/EBPβ to activate transcription at the promoters for C/EBPα and c-*fos*. Different C/EBPβ-responsive promoters require different patterns of acetylated lysines in C/EBPβ for transcription activation. Further, C/EBPβ acetylation was increased by growth hormone (GH), and mutation of K39 impaired GH-stimulated c-*fos* promoter activation. These data suggest that acetylation

of K39 of C/EBPβ, alone or in combination with acetylation at other lysines, may play a role in C/EBPβ-mediated transcriptional activation.

Introduction

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 (1, 2). It is now recognized that many cellular proteins are acetylated. In the nucleus, acetylation of several transcription factors is reported to have broad impact on their function. For example, acetylation of p53 stabilizes it by preventing its ubiquitination by Mdm2, allowing p53 to enter the nucleus to activate target genes (3). Acetylation of p53 at lysines 320, 373, and 382 increases its binding to cognate DNA (4, 5). Acetylation is also reported to increase nuclear localization of NF-κB (6), which is essential for its transcription factor function. Acetylation of GATA-1 was found to increase its binding to DNA, thereby stimulating GATA-1 dependent transcription (7). Other functional consequences of acetylation include promoting interaction of the nuclear import factor importin-α with importin-β (8). Ku70 is unable to bind and sequester pro-apoptotic BAX when Ku70 is acetylated (9). Acetylation in the DNA-binding domain of HMGI(Y) is inhibitory, decreasing its DNA-binding ability and weakening its transcriptional potency (10). Thus, acetylation modifies the function of a variety of cellular proteins.

CCAAT/Enhancer Binding Protein (C/EBP) β is a B-ZIP transcription factor that is expressed in adipose, hepatic, immune and a variety of other tissues (11-16). C/EBPβ

is present in cells in three forms: Full length Liver-enriched Activating Protein 1 (LAP1, residues 1-296) (17), LAP2 (22-296), and the inhibitory form LIP (151-296). C/EBPβ plays an important role in the gluconeogenic pathway (18), liver regeneration (19), and the hematopoietic system (20). Among the many genes responsive to C/EBPβ, the proto-oncogene c-*fos* is one prominent example (21, 22). As other examples, C/EBPβ, an early mediator of the differentiation of adipocytes (15, 23-25), is well-known to activate expression of genes for C/EBPα and PPARγ, which in turn mediate adipogenic differentiation (25-28).

The function of C/EBPβ is modulated by its phosphorylation at several sites. For example, phosphorylation of human C/EBPβ at a Mitogen-Activated Protein Kinase (MAPK) substrate site at T235 (which corresponds to T188 in mouse C/EBPβ) (29) alters its ability to activate transcription of a variety of downstream target genes including c-*fos* (21, 30). The phosphorylation of C/EBPβ at T188 is rapidly increased in an Extracellular signal Regulated Kinase (ERK)-dependent manner by factors such as growth hormone (GH) (30), interleukin-1 β (31) and interferon-γ (32). Phosphorylation of C/EBPβ at T188 is also increased during adipogenesis in preadipocytes and NIH-3T3 cells (33-35). Murine C/EBPβ has been observed to relocalize to heterochromatin within the nucleus in response to GH in a manner dependent on its phosphorylation at T188 (36). Phosphorylation of rat C/EBPβ at S105 or of mouse C/EBPβ at T217 by p90 ribosomal S6 kinase stimulates proliferation in differentiated hepatocytes induced by TGFα (37). Protein Kinase A or Protein Kinase C-mediated phosphorylation at S240 of rat C/EBPβ is reported to attenuate DNA binding (38). Further, GH induces a delayed de-phosphorylation at a GSK-3 site at S184 of mouse C/EBPβ, which may interfere with its

binding to the c-*fos* promoter (39). Phosphorylation of C/EBPβ at S184 also contributes to adipogenesis and activation of adipocyte genes such as C/EBPα and aP2 (35).

C/EBPβ has been shown to interact with p300 (40), a nuclear co-activator with intrinsic acetyltransferase activity (41). C/EBPβ also associates with cyclic-AMP response element binding protein (CREB) Binding Protein (CBP), a coactivator and acetyltransferase homologous to p300 (42, 43). Because of the association between C/EBPβ and these acetyltransferases, this study investigates the acetylation of C/EBPβ and its functional consequences. The present report indicates that C/EBPβ is acetylated, in agreement with several recent reports (44, 45). Multiple acetylation sites are identified, including a novel acetylation site on C/EBPβ at lysine 39 (K39) (numbering is based on the murine sequence of C/EBPβ unless indicated otherwise). Importantly, mutation of K39 decreases the ability of C/EBPβ to mediate transcriptional activation of target gene promoters. Using an anti-acetyl-lysine antibody that recognizes acetylated K39, endogenous C/EBPβ was found to be acetylated in preadipocytes, in which C/EBPβ is an important factor during adipogenesis. C/EBPβ is a critical mediator of GH-regulated transcription of c-*fos* (46, 47). A non-acetylatable mutation at K39 of C/EBPβ impaired GH-stimulated c-*fos* promoter activation, and GH was found to increase acetylation of C/EBPβ. Taken together, this study identifies acetylation at K39 as novel modification of C/EBPβ that is regulated and contributes, alone and in combination with other acetylatable lysines, to C/EBPβ-mediated transcription.

Materials and Methods

Plasmids and antibodies

The numbering used to designate residues in C/EBPβ is based on the mouse C/EBPβ sequence (Accession # NM009883). The following C/EBPβ plasmids were used: The plasmid encoding full-length C/EBPβ (LAP1) driven by the CMV promoter (CMV-C/EBPβ) was a gift from Dr. U. Schibler (University of Geneva) and Dr. L. Sealy (Vanderbilt University). The plasmid HA-C/EBPβ encodes C/EBPβ (residues 22-296, also known as LAP2) tagged with HA at the N-terminus (42). HA-C/EBPβ is able to activate the c-*fos* promoter at least as well as full-length CMV-C/EBPβ (data not shown). LAP2 is a prominent active form of C/EBPβ in GH-responsive 3T3-F442A cells (21). Hereafter, C/EBPβ will be designated as LAP2 unless otherwise indicated. Mutations were introduced into HA-C/EBPβ at indicated residues using Stratagene QuikChange XL Site Directed Mutagenesis Kit. All mutations were confirmed by sequencing. The mutated forms of C/EBPβ are referred to as: K39R, K39A, K39Q, K117R, K215/216R; TM refers to combined mutations K39, 117, 215/216R. As additional controls for mutation of K39 in the transcriptional activation domain of C/EBPβ, arginine 42 was mutated to alanine (R42A) and lysine 98 was mutated to arginine (K98R). HA-C/EBPβ mutated to alanine at phosphorylation sites T188 (T188A) or S184 (S184A) were similarly generated. GST-C/EBPβ plasmids encode fusion proteins of GST with truncated forms of C/EBPβ (residues 22-227, 22-193, 22-103) as previously described (42). HIS-C/EBPβ encodes C/EBPβ tagged with six histidine residues at the N-terminus (48). Recombinant HIS-C/EBPβ was expressed and purified on a Ni-NTA agarose

column (Qiagen).

Plasmids for full-length, N-terminally flag-tagged p300 (p300) and N-terminally flag-tagged P/CAF (P/CAF) were prepared, expressed and purified as previously described (49, 50). The plasmid 5XC/EBP-luc encodes a luciferase reporter gene driven by five copies of a consensus C/EBP site (42). The plasmid C/EBPα-luc was a gift from Dr. O. MacDougald (University of Michigan) (34). The plasmid for c-*fos*/luciferase (c-*fos*-luc), which contains the mouse c-*fos* promoter (–379 to +1) upstream of luciferase, was a gift from Dr. W. Wharton (University of S. Florida) and Dr. B. Cochran (Tufts University) (51). A plasmid encoding rat growth hormone receptor (GHR) was provided by Dr. C. Carter-Su (University of Michigan) (52). RSV-β-galactosidase (Bgal) was provided by Dr. M. Uhler (University of Michigan). pcDNA3.1 vector, used to normalize the total amount of DNA in transfections, was purchased from Clontech.

The following antibodies were used: anti-HA (Covance) and anti-C/EBPβ (Santa Cruz, specific for the C-terminus of C/EBPβ) were used at dilutions of 1:100 for immunoprecipitations and 1:1000 for immunoblotting. Anti-acetyl-lysine (anti-Ac-K, Upstate, monoclonal antibody 4G12 which detects acetylated lysines on histones and p53) was used at a dilution of 1:500 for immunoblotting. An antibody against a peptide corresponding to human C/EBPβ phosphorylated at T235 (homologous to T188 of mouse C/EBPβ) (anti-pC/EBPβ, Cell Signaling) was used at a dilution of 1:1000 for blots.

Cell culture

293T cells were provided by Dr. M. Lazar (University of Pennsylvania). Murine 3T3-F442A preadipocyte fibroblasts were provided by Dr. H. Green (Harvard University) and

Dr. M. Sonenberg (Sloan-Kettering). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 8% calf serum (Invitrogen) in an environment of 10% CO_2/90% air at 37°C. Prior to use in experiments, cells were incubated overnight in serum-free DMEM containing 1% bovine serum albumin (BSA, CRG7, Serological Corp), which was also supplemented with deacetylase inhibitors trichostatin A (TSA, 1μM, Sigma) and nicotinamide (NAM, 5mM, Sigma). Chinese Hamster Ovary cells expressing rat GH receptor (GHR) containing the N-terminal half of the cytoplasmic domain (referred to as CHO-GHR cells) were provided by Dr. G. Norstedt (Karolinska Institute, Stockholm, Sweden) and Dr. N. Billestrup (Steno Diabetes Center, Copenhagen, Denmark) (53). They were maintained in Ham's F12 Medium (Gibco/Invitrogen) containing 8% fetal bovine serum and 0.5mg/ml Geneticin (Gibco/Invitrogen) in 5%CO_2/95% air at 37°C. Prior to experiments, CHO-GHR cells were incubated overnight in medium containing 1% BSA instead of serum. All media were supplemented with 1mM L-glutamine, 100units/ml penicillin, 100μg/ml streptomycin, and 0.25μg/ml amphotericin. Calcium phosphate transfections were performed as previously described (54), except 50mM HEPES-buffered saline was used instead of BES-buffered saline.

Immunoprecipitation and immunoblotting

293T cells were lysed using Lysis Buffer (420 mM NaCl, 20mM Hepes (pH 7.9), 1mM EDTA, 1mM EGTA, 20% glycerol), supplemented with 150mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 10μg/ml aprotinin and leupeptin, as well as 1μM TSA and 5mM NAM. For 293T cells expressing HA-C/EBPβ,

lysates were precleared using protein G sepharose beads (G beads, Amersham). Samples were immunoprecipitated using anti-HA antibody for 2 hr at 4°C, and immunoprecipitates were collected on beads for 1 hr. To test endogenous proteins, 3T3-F442A cells were lysed in SDS lysis buffer (50mM Tris-HCl, 1% SDS, 10mM EDTA, 1mM EGTA, 0.2% Triton X-100). Lysates were precleared using protein A agarose beads (A beads, RepliGen). Samples were immunoprecipitated using anti-C/EBPβ antibody for 3 hr at 4°C, and immunoprecipitates were collected on beads for 1 hr. Beads were washed three times in Acetylase Buffer (10mM Tris, pH 7.6, 150mM NaCl, 1mM EDTA, and 5% glycerol, 22 mg/ml sodium butyrate (Sigma), 3 mg/ml dithiothreitol (Invitrogen)). SDS protein dye (50mM Tris, 1% SDS, 0.001% Bromophenol blue, 10% glycerol, 10% β-mercapo-ethanol) was then added to the beads and samples were boiled, separated by SDS-PAGE, and immunoblotted as previously described (55). Bands on immunoblots were visualized using IRDye 700-coupled antimouse IgG (1:10,000) or IRDye 800-coupled antirabbit IgG (1:10,000) on the Odyssey infrared scanning system (LI-COR, Inc., Lincoln, NE) as previously described (47). Molecular weight was estimated using Kaleidoscope protein molecular weight standard (Biorad).

In vitro acetylation of C/EBP*β*

Acetylation assays were performed using Acetylase Buffer. To test the acetylation of C/EBPβ *in vitro*, purified HIS-C/EBPβ (3μg) or the purified GST-C/EBPβ fusion proteins (3μg each) were incubated in Acetylase Buffer alone or with added purified p300 or P/CAF (1μg each), and 1μl [^{14}C]acetyl-CoA (55mCi/mmol, ICN). Samples were incubated for 1 hr at 30°C, separated by SDS-PAGE (8%), and analyzed by

autoradiography (Kodak X-Omat Blue XB-1). To examine acetylation of expressed C/EBPβ, CMV-C/EBPβ (LAP1) was expressed in 293T cells to obtain high protein expression. C/EBPβ was immunoprecipitated using anti-C/EBPβ, and used in the acetylase assay described above. Protein levels were assessed by staining the gel with Coomassie brilliant blue G-250 (coomassie, Biorad). For expressed CMV-C/EBPβ, a duplicate immunoblot was probed with anti-C/EBPβ to evaluate migration.

C/EBPβ peptide acetylation

The following peptides containing candidate lysines in C/EBPβ were synthesized at the University of Michigan Protein Core: DCLAYGAKAARAAPR (amino acids (aa) 32-46), FADDGAKPSKKPADYGYV (aa 91-109), SLGRAGAKAAPPACF (aa 110-124), PPPPALLKAEPGFE (aa 126-139), GFEPADCKRADDPA (aa 137-151), PSPADAKAAPAACF (aa 189-202), PAAPAKAKAKKTVDKLSD (aa 206-223). A peptide based on histone H3 (aa 7-22) that is acetylated by p300 and P/CAF (50) served as a positive control: ARKSTGGKAPRKQLAT. Each peptide (2μg) was incubated in Acetylase Buffer with 1 μl [^{3}H] acetyl-CoA (5.8 Ci/mmol, ICN), without or with purified p300 or P/CAF (1μg each), and incubated for 1 hr at 30°C. Samples were then spotted on grade P81 cellulose paper (Whatman). Paper was rinsed with 0.1% phosphoric acid (Sigma) and acetylation, expressed as relative CPM, was measured in CytoScint scintillation cocktail (ICN Biomedicals) using a liquid scintillation counter (Packard).

In vivo acetylation of C/EBP*β*

CMV-C/EBPβ (LAP1) was expressed in 293T cells and 48 hr later cells were incubated

with 200 μl of [^{3}H]sodium acetate (2.90 Ci/mmol, ICN) for 1 hr. C/EBPβ was immunoprecipitated with antibodies against C/EBPβ or rabbit IgG (Sigma), which served as a control. Samples were separated by SDS-PAGE (8%), and analyzed by autoradiography.

To assess the acetylation of WT HA-C/EBPβ or of HA-C/EBPβ mutated at various lysines, appropriate plasmids were co-expressed with or without plasmids for p300 (2.2ug) or P/CAF (0.2ug) in 293T cells. pcDNA3 was used to control for total amount of DNA transfected. 24 hrs later, cells were incubated overnight with TSA (1μm) and NAM (5mM) in serum-free DMEM containing 1% BSA. Cell lysates were subjected to immunoprecipitation with anti-HA, samples were separated by SDS-PAGE (4-20%), transferred to PVDF membrane, and probed with either anti-Ac-K or anti-HA. Phosphorylation of WT or mutated HA-C/EBPβ was similarly analyzed, except immunoblots were probed with anti-pC/EBPβ instead of anti-Ac-K.

To examine the regulation of acetylation of C/EBPβ, 293T cells were transfected with plasmids for WT HA-C/EBPβ and GHR, and then 24 hrs later, incubated overnight with TSA (1μm) and NAM (5mM) in serum-free DMEM containing 1% BSA. 48 hrs after transfection, cells were treated with 250 ng/ml (11.5 nM) human GH (recombinant GH kindly provided by Eli Lilly, Inc.) for 15 min prior to lysis. Cells were lysed in Lysis Buffer, samples were immunoprecipitated with anti-HA as described, and analyzed by immunoblotting with anti-Ac-K and anti-C/EBPβ. Membranes were scanned using the Odyssey infrared scanning system (47), and bands were quantified using Odyssey software. Acetylation of C/EBPβ was calculated using values for acetylation (anti-Ac-K) divided by total C/EBPβ (anti-C/EBPβ or anti-HA). Statistical analysis of results from

three or more experiments was performed using Student's t-test (Excel) or 1-way ANOVA and Bonferroni's Multiple Comparison Test (Prism version 3).

Transcription assays

WT HA-C/EBPβ or HA-C/EBPβ mutated at various residues alone or in combination (each 400 ng/35mm well) were co-expressed with reporter plasmids 5X-C/EBP-luc, C/EBPα-luc, or c-*fos*-luc each (400ng/well) in CHO-GHR cells, a reliable system to assess reporter gene activation with or without GH. Bgal (300ng/well) was co-expressed to normalize for transfection efficiency. 24 hr after transfection, cells were deprived of serum and lysed for luciferase asssay 24 hr after that, as previously described (56). In some experiments, cells were treated with vehicle or GH (500ng/ml, 23 nM) for 4 hr before lysates were prepared. Transcriptional activation was determined by luciferase output as measured using an Opticomp Luminometer, and is expressed as relative luciferase units/Bgal (RLU). Each condition was analyzed in triplicate for each experiment. Statistical analysis of results from replicate, independent experiments was performed using 1-way ANOVA and Bonferroni's Multiple Comparison Test (Prism version 3; www.GraphPad.com).

Results

C/EBPβ is acetylated in vitro and in vivo

Since C/EBPβ and several acetyltransferases interact to activate transcription (40, 42, 47), the acetylation of C/EBPβ was examined. The ability of p300 or P/CAF to acetylate C/EBPβ was tested *in vitro* using purified C/EBPβ in the presence of purified p300 or

P/CAF and [^{14}C]acetyl-CoA. A prominent labeled band (Fig 2.1A, upper panel, arrowhead) was detected when p300 or P/CAF was present, but not in their absence. Additional evidence suggesting that C/EBPβ is acetylated was obtained by expressing C/EBPβ in 293T cells and subjecting lysates to immunoprecipitation with antibodies against C/EBPβ. When immunoprecipitates were incubated with [^{14}C]acetyl-CoA in the presence of purified p300 or P/CAF, label was incorporated into bands (Fig 2.1B, upper panel, arrowhead) which co-migrate with C/EBPβ (Fig 2.1B, lower panel), indicating that C/EBPβ is acetylated by both p300 and P/CAF.

To determine if C/EBPβ is acetylated *in vivo*, 293T cells expressing C/EBPβ were incubated with [^{3}H] sodium acetate and C/EBPβ was immunoprecipitated using anti-C/EBPβ. A labeled band (Fig 2.2A, arrowhead) migrating at a size that corresponds to C/EBPβ (data not shown) suggests that C/EBPβ is also acetylated *in vivo*. In agreement, an antibody specific for acetylated lysine (anti-Ac-K) detected acetylation of C/EBPβ *in vivo*. When HA-C/EBPβ was expressed in 293T cells and C/EBPβ was immunoprecipitated using anti-HA, basal acetylation of C/EBPβ was detected on immunoblots using anti-Ac-K (Fig 2.2B). The acetylation of C/EBPβ increased when p300 was co-expressed, which is consistent with the *in vitro* acetylation observed.

Acetylation of endogenous C/EBPβ is detected using the anti-Ac-K antibody when C/EBPβ is immunoprecipitated from lysates of 3T3-F442A preadipocytes using anti-C/EBPβ (Fig 2.2C, lane 3, arrowhead), but not in controls incubated without antibody or with IgG (lanes 1 and 2, respectively). These results indicate that endogenous C/EBPβ in 3T3-F442A cells is acetylated.

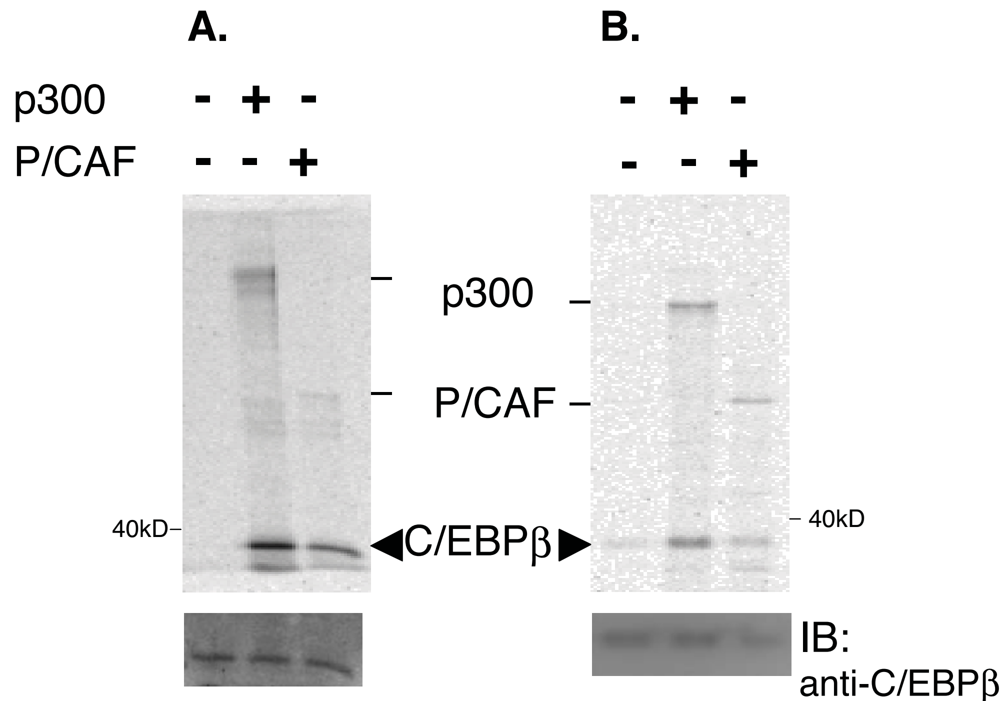


Fig 2.1. C/EBPβ is acetylated *in vitro*. **A**, Purified HIS-C/EBPβ was incubated alone, with purified p300 or purified P/CAF as indicated, in the presence of [^{14}C]acetyl-CoA. Autoradiograph (upper panel) shows acetylation of C/EBPβ (arrowhead) in the presence of p300 and P/CAF. Migration of auto-acetylated p300 and P/CAF are indicated by dashes on the right. Lower panel shows protein labeled with Coomassie Blue to indicate loading of C/EBPβ in all lanes. Apparent molecular weight is indicated in the margins. Similar results were obtained in two other experiments. **B**, CMV-C/EBPβ expressed in 293T cells was immunoprecipitated using anti-C/EBPβ and used in an acetylation assay without or with purified p300 or P/CAF. Acetylated C/EBPβ (arrowhead) and autoacetylated p300 and P/CAF (dashes on left) are shown in the autoradiograph (upper panel). Immunoblot probed with anti-C/EBPβ indicates expression of C/EBPβ (lower panel). Similar results were obtained in two other experiments.

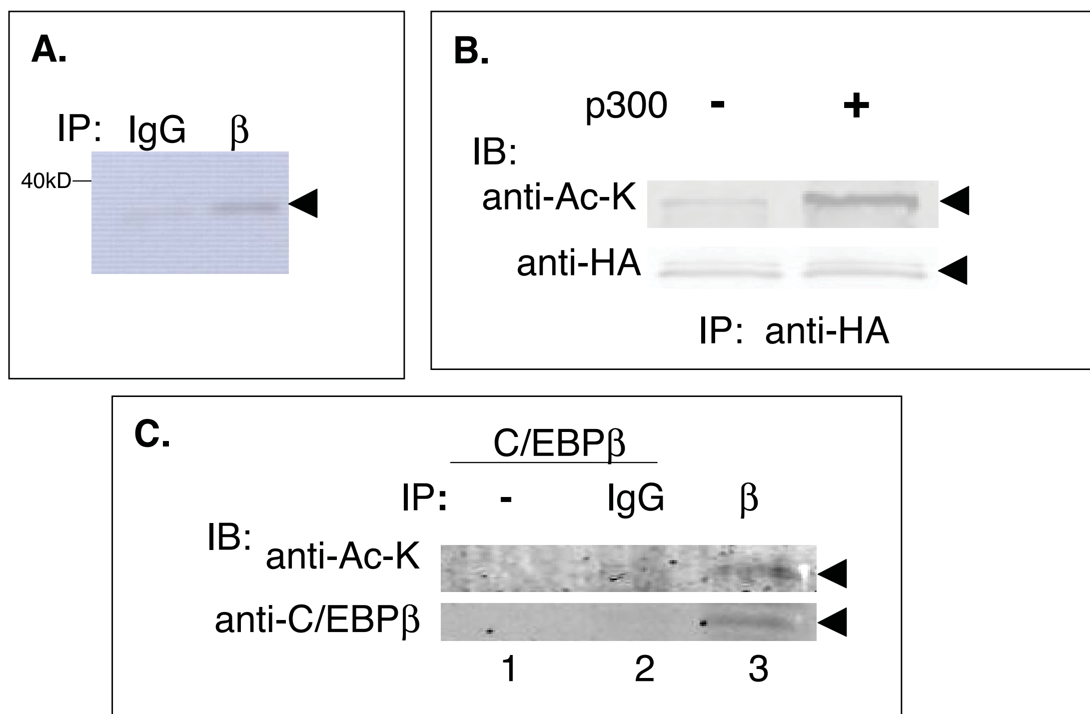


Fig 2.2. C/EBPβ is acetylated *in vivo*. **A**, 293T cells expressing CMV-C/EBPβ were incubated with [^{3}H]-acetate. Cell lysates immunoprecipitated using anti-C/EBPβ (β) or rabbit IgG (IgG) are shown in an autoradiograph. Arrowhead indicates acetylated C/EBPβ. **B**, WT HA-C/EBPβ was expressed in 293T cells in the absence (-) or presence (+) of p300. Cell lysates were subjected to immunoprecipitation using anti-HA, and used for immunoblotting with either anti-Ac-K (upper panel) or anti-HA (lower panel). Similar results were obtained in two other experiments. ***C***, Endogenous C/EBPβ was immunoprecipitated from 3T3-F442A preadipocytes with anti-C/EBPβ. Samples incubated without antibody (lane 1) or with IgG (lane 2) served as controls. Samples were used for immunoblotting with anti-Ac-K (upper panel, arrowhead) or anti-C/EBPβ (lower panel). Similar results were obtained in three experiments.

C/EBPβ contains multiple acetylation sites

Full-length murine C/EBPβ (LAP1) is a 296-residue protein that contains 21 lysines (Fig 2.3A). The acetylation of several forms of truncated C/EBPβ (residues 22-227, 22-193, and 22-103) (bottom of Fig 2.3A) fused to GST was tested. The GST-C/EBPβ fusion proteins were incubated *in vitro* with [^{14}C] acetyl-CoA and p300 or P/CAF (Fig 2.3B). Label was incorporated into all three forms of GST-C/EBPβ, in the presence of p300 (Fig 2.3B, lanes 2,5,8) or P/CAF (lanes 3,6,9), but not in their absence (lanes 1,4,7). The acetylation of GST-C/EBPβ 22-227 agrees with a previous report that C/EBPβ is acetylated at lysines 215/216 (45). However, the shorter truncations of GST-C/EBPβ (22-193 and 22-103), which do not contain K215 or K216, were also acetylated. The acetylation decreased progressively with shorter truncations. These finding agree with previous observations of acetylation at K215/216 (45), and indicate that additional acetylation sites exist within the C/EBPβ molecule. Thus, C/EBPβ is acetylated at multiple lysines.

C/EBPβ is acetylated at lysine 39 in its transcription activation domain

C/EBPβ contains an N-terminal transcription activation domain, C-terminal DNA binding and dimerization domains, and an intervening repression domain (15, 17, 60-63). As a first approach to examine which lysines within C/EBPβ are acetylated, seven peptides corresponding to C/EBPβ sequences containing candidate lysines primarily in transcription regulatory domains (Fig 2.3A) were synthesized and their acetylation was determined *in vitro* using [^{14}C]acetyl-CoA and p300 or P/CAF (Fig 2.4A). Peptides

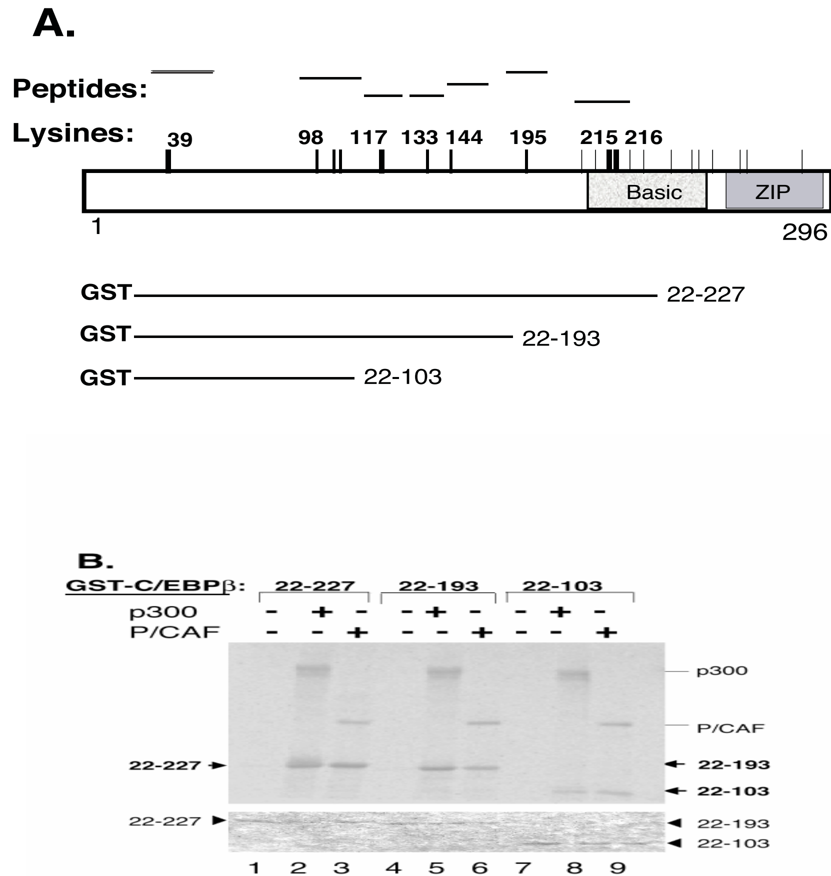


Fig 2.3. C/EBPβ is acetylated at multiple lysines. **A**, Diagram of the sequence of C/EBPβ: The transcriptional activation domain lies at the N-terminus, basic region mediates DNA binding, and leucine zipper (ZIP) mediates dimerization. Horizontal bars above the diagram indicate position of synthetic C/EBPβ peptides containing candidate lysines that were tested. Vertical lines above diagram indicate lysines in C/EBPβ; for reference, some lysine residues represented in peptides are numbered. GST-C/EBPβ fusion proteins are diagrammed schematically below. **B**, Purified GST-C/EBPβ 22-227 (lanes 1-3), GST-C/EBPβ 22-193 (lanes 4-6), or GST-C/EBPβ 22-103 (lanes 7-9) was incubated alone (lanes 1,4,7), with p300 (lanes 2,5,8) or P/CAF (lanes 3,6,9), in the presence of [^{14}C] acetyl-CoA. Autoradiograph (upper panel) shows acetylation of each form of acetylated GST-C/EBPβ (arrows). Auto-acetylated p300 and P/CAF are indicated by dashes on the right. Coomassie Blue staining (lower panel) verified appropriate migration and comparable loading of GST fusion proteins (arrowheads). Similar results were obtained in two other experiments.

corresponding to lysines 39, 117, and 215/216 of C/EBPβ were acetylated in the presence of p300 (black bars). The C/EBPβ peptide containing lysines 215 and 216 also contains three other lysines at positions 211, 213, and 220; the acetylation sites in the peptide are referred to here as K215 and K216 based on a previous report that these two lysines of C/EBPβ are acetylated (45). Interestingly, p300, but not P/CAF, acetylated these peptides, although P/CAF as well as p300 increased acetylation of a histone H3 peptide control.

To test whether the candidate lysines within the C/EBPβ molecule are acetylated, lysines 39, 117, and the combination of 215 and 216 were mutated within HA-C/EBPβ to non-acetylatable arginine residues (3, 64), alone or in combination (Fig 2.4B). WT HA-C/EBPβ or each of its mutated forms was expressed without or with p300 or P/CAF in 293T cells. C/EBPβ in cell lysates was immunoprecipitated with anti-HA and immunoblotted with anti-Ac-K. Acetylation of WT C/EBPβ was slightly visible in the absence of HATs (lane 3), and the ability of p300 or P/CAF to increase acetylation of WT C/EBPβ is clearly evident (lanes 4,5). In contrast, when K39 was mutated, acetylation of C/EBPβ was almost completely obliterated, even in the presence of p300 or P/CAF (lanes 6-8). On the other hand, acetylation of K117R C/EBPβ (lane 10,11) and K215/216R C/EBPβ (lanes 13,14) was induced by p300 and P/CAF comparably to WT C/EBPβ. These findings suggest that anti-Ac-K detects acetylation of C/EBPβ at K39, but not at the other sites tested. Further, when K39 was mutated in combination with K117, K215, and K216 (TM, lanes 15-17), acetylated C/EBPβ was also undetectable. Mutation of K39 of C/EBPβ in various combinations with the other candidate lysines

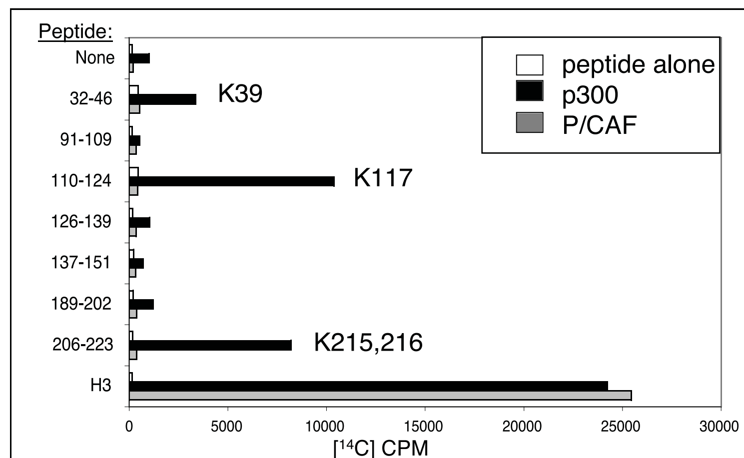
(K39R/117R, K39/215/216R, or K39/117/215/216R) also prevented acetylation, even in the presence of p300 (Supplemental Fig 2.1).

The specificity of the decrease in acetylation of K39 C/EBPβ was assessed by several other comparisons. Since K39 lies in he transcriptional activation domain, it is relevant that mutations at nearby arginine 42 (R42A, lane 21) and at lysine 98 (K98R, lane 22), the nearest lysine residue to K39, which all lie in the transcriptional activation domain of C/EBPβ, did not interfere with acetylation as K39R did (lane 20). This suggests that mutation at K39 does not simply disrupt the integrity of the transcriptional activation domain. Further, mutation K39A C/EBPβ (lane 26), like K39R (lane 25), also disrupted acetylation. These studies indicate that K39 of C/EBPβ is acetylated, and support the specificity of acetylation of C/EBPβ at K39. These findings also suggest that the anti-Ac-K antibody used may detect acetylation only at K39 among the multiple sites tested in C/EBPβ. The apparent specificity of anti-Ac-K for K39 and the fact that K39 lies in the transcriptional activation domain of C/EBPβ led to selection of K39 as an acetylation site in C/EBPβ that warranted further analysis.

C/EBPβ is acetylated at K39 despite mutation at phosphorylation sites T188 or S184

Acetylation has been linked to phosphorylation for several proteins. For example, prior phosphorylation of histone H3 at serine 10 is required for its acetylation at lysine 14 (65-67). C/EBPβ is phosphorylated at several sites, including T188, which is a substrate for ERK1/2, and S184, which is a substrate of GSK-3 (29, 30, 35, 37, 39, 68). The possible dependence of acetylation of C/EBPβ on its phosphorylation was examined by expressing WT C/EBPβ, or C/EBPβ with mutations in two of the regulated

A.



B.

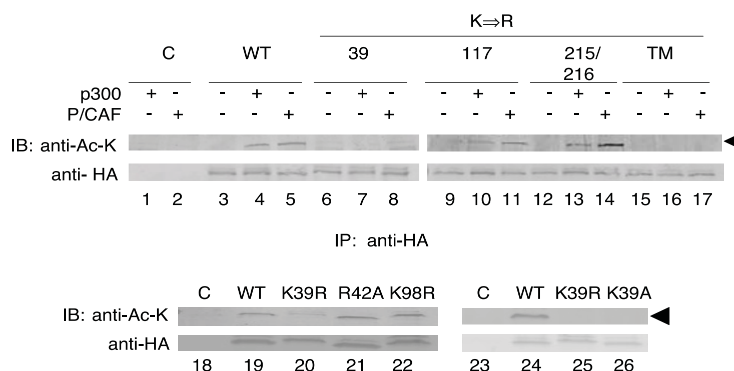


Fig 2.4. C/EBPβ is acetylated at lysine 39. A, The indicated peptides (left) containing candidate lysines in C/EBPβ were incubated alone (open bars), with p300 (black bars), or P/CAF (grey bars). Bars represent relative incorporation of labeled [^{14}C] acetyl-CoA as CPM. Candidate lysines (K) within acetylated peptides are marked next to relevant bars. Peptide based on histone H3 was used as a positive control. Similar results were obtained in two other experiments. **B**, Plasmids for HA-C/EBPβ without (WT) or with mutations (K⇒R) at indicated lysines, alone or in combination (TM=K39/117/215/216R), were co-expressed with pcDNA3 control vector, p300, or P/CAF in 293T cells (upper pair of panels). Similarly, plasmids for HA-C/EBPβ without (WT, lanes 19,24) or with mutations at K39 (K39R and K39A, lanes 25-26), R42 (R42A) or K98 (K98R) (lanes 21,22) or pcDNA3 control (lanes 18,23) were expressed in the presence of p300 (lanes 18-26). C/EBPβ was immunoprecipitated with anti-HA and used for immunoblotting with anti-Ac-K (upper panel) or anti-HA (lower panel). Arrowheads on the right indicate migration of acetylated C/EBPβ. Similar results were obtained in two other experiments.

phosphorylation sites, S184 or T188, with or without p300. C/EBPβ was immunoprecipitated with anti-HA and immunoblotted with anti-Ac-K to determine whether mutating phosphorylation sites of C/EBPβ would alter its acetylation at K39 (Fig 2.5). Basal acetylation of WT C/EBPβ was slightly detectable (lane 2), and increased in the presence of p300 (lanes 3), as expected. Mutating S184 (lane 4) or T188 (lane 6) in C/EBPβ did not impair basal acetylation of K39 as detected by anti-Ac-K. Further, acetylation of C/EBPβ mutated at these phosphorylation sites increased in the presence of p300 as it does as for WT C/EBPβ (lanes 5,7). Blotting with an antibody specific for C/EBPβ phosphorylated at T188 (anti-pC/EBPβ) indicates that WT, S184 and TM C/EBPβ, but not T188A C/EBPβ, were phosphorylated at T188. Phosphorylation of WT and S184A C/EBPβ at T188 was similar in the absence or presence of p300 (middle panel, lanes 2-5). These findings indicate that for the sites and conditions tested, C/EBPβ is acetylated at K39 even when phosphorylation at S184 or T188 in C/EBPβ is prevented by mutation. Conversely, anti-pC/EBPβ recognizes WT C/EBPβ, and mutation of C/EBPβ at candidate acetylation sites at K39, K117 or K215/216, alone or in combination did not alter its phosphorylation at T188 compared to WT C/EBPβ (Supplemental Fig 2.2). Further, phosphorylation of C/EBPβ was not detectably altered by p300 for WT C/EBPβ or any of the acetylation site mutants. Together, these data suggest that acetylation of C/EBPβ at K39 and its phosphorylation at T188 are independent of each other.

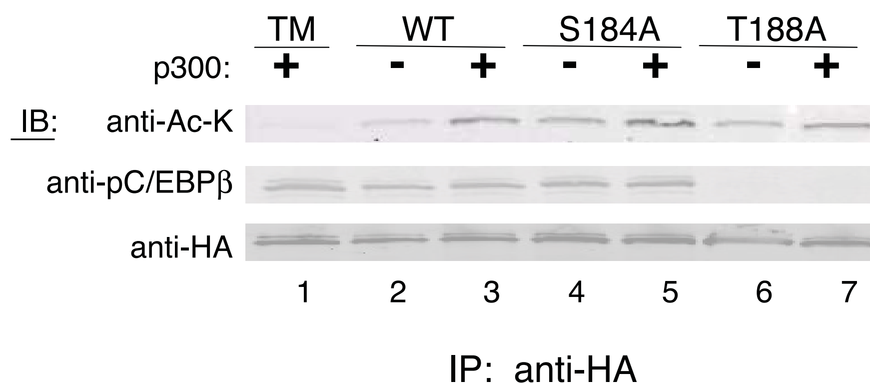


Fig 2.5. C/EBPβ is acetylated at lysine 39 when phosphorylation sites T188 and S184 are mutated. Plasmid for WT HA-C/EBPβ (lanes 2-3) or HA-C/EBPβ with alanine mutations at phosphorylation sites S184 (lanes 4-5) or T188 (lanes 6-7) was expressed without (lanes 2,4,6) or with p300 (lanes 3,5,7) in 293T cells. C/EBPβ was immunoprecipitated with anti-HA and used for immunoblotting with anti-Ac-K (upper panel), anti-pC/EBPβ (middle panel), or anti-HA (lower panel). TM HA-C/EBPβ (mutated to R at K39, 117, 215 and 216) co-expressed with p300 (lane 1) served as a negative control for acetylation. Similar results were obtained in two other experiments. Quantification using Odyssey software was determined by anti-Ac-K normalized to anti-HA. WT C/EBPβ was significantly less than WT C/EBPβ + p300 ($p < 0.001$), S184A C/EBPβ was significantly less than S184A C/EBPβ + p300 ($p < 0.05$), and T188A C/EBPβ was significantly less than T188A C/EBPβ + p300 ($p < 0.001$). WT C/EBPβ was not significantly different from S184A C/EBPβ or T188A C/EBPβ. WT C/EBPβ + p300 was not significantly different from S184A C/EBPβ + p300 or T188A C/EBPβ +p300.

Mutation of K39 in C/EBPβ impairs its ability to activate transcription at a consensus C/EBP site

K39 of C/EBPβ lies within its transcriptional activation domain (69). To test whether K39 of C/EBPβ contributes to its ability to activate transcription, activation of a consensus C/EBP site (5XC/EBP-luc) by WT C/EBPβ or K39R C/EBPβ was compared in CHO-GHR cells (Fig 2.6A). WT C/EBPβ significantly increased promoter activity compared to control cells transfected with vector alone, as expected (42). Mutation K39R completely blocked the ability of C/EBPβ to mediate transcriptional activation via the consensus C/EBP site. The specificity of the decrease in activation by K39R C/EBPβ was assessed by comparing C/EBPβ mutated at nearby arginine 42 (R42A) or at lysine 98 (K98R), which also lie in the transcriptional activation domain of C/EBPβ. R42A and K98R C/EBPβ increased promoter activity to the same extent as WT C/EBPβ, consistent with the observation that they are acetylated comparably to WT C/EBPβ (Fig 2.4B). Expression of the various forms of C/EBPβ was comparable (Fig 2.6A, lower panel), indicating that the decrease in promoter activation by K39R C/EBPβ does not reflect a difference in expression. These findings indicate that impairment of acetylation as well as impaired transcriptional activation by mutation of K39 in C/EBPβ is specific for K39 and do not reflect a disabled transcriptional activation domain. Mutating K39 to alanine (A) decreased C/EBPβ-mediated transcriptional as effectively as K39R (Fig 2.6B), indicating that the lysine *per se*, rather than the charge, determines the function of C/EBPβ (50). This is also consistent with a lack of acetylation of both K39A and K39R C/EBPβ (Fig 2.4B). Conversely, mutating lysine to glutamine (Q) is thought to mimic

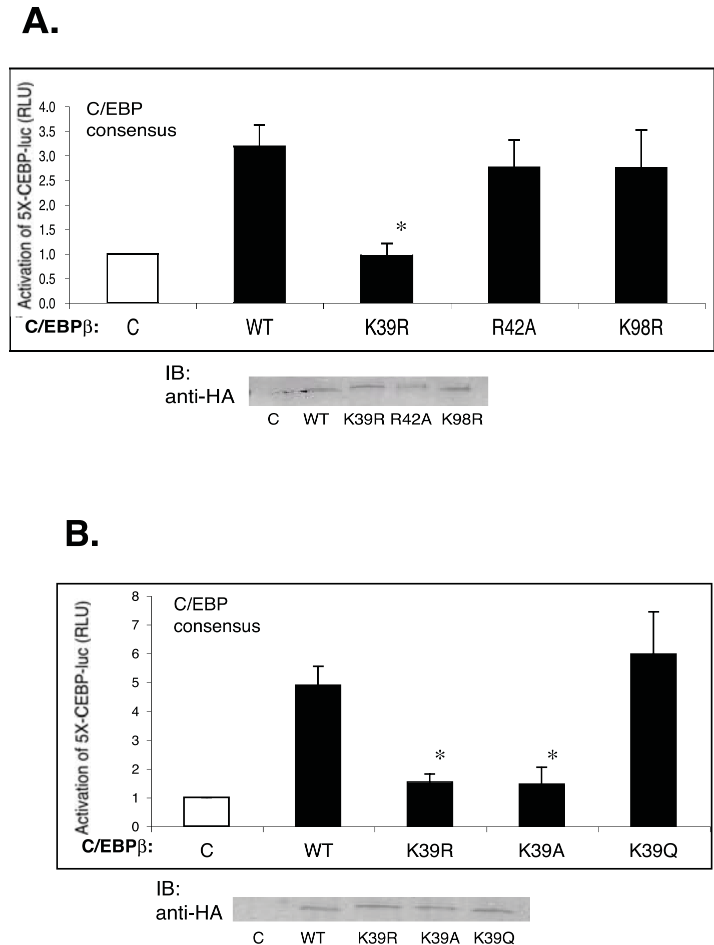


Fig 2.6. Mutations of K39 of C/EBPβ impair transcriptional activation of a consensus C/EBP site. **A**, Plasmids for WT HA-C/EBPβ, K39R, R42A, K98R C/EBPβ, or pcDNA3.1 vector (C) were co-expressed with 5XC/EBP-luc in CHO-GHR cells. Cells were lysed 48 hr later and luciferase activity was measured. Transcriptional activation (RLU) is expressed relative to C=1 and bars represent mean + SEM in this and subsequent figures. SEM of control is too small to be visible (n=3 independent experiments). A representative immunoblot of lysates used for luciferase assay, probed with anti-HA, shows relative expression of C/EBPβ (lower panel in this and subsequent figures). Activation in the presence of K39R is significantly less ($p < 0.05$, asterisk) than with WT C/EBPβ. Transcriptional activation by WT C/EBPβ is significantly greater ($p < 0.05$) than control, and activation by R42A and K98R C/EBPβ is not significantly different from WT C/EBPβ. **B**, The plasmid for 5XC/EBP-luc was co-expressed with plasmids for C/EBPβ without (WT) or with the indicated mutations of K39 and luciferase activity was measured (n=3 experiments). Activation of 5XC/EBP-luc is significantly lower ($p < 0.001$, asterisks) with K39R and K39A than WT C/EBPβ. Transcriptional activation of 5XC/EBP-luc by WT C/EBPβ is significantly greater ($p < 0.001$) than control, and activation by K39Q C/EBPβ is not significantly different from WT C/EBPβ.

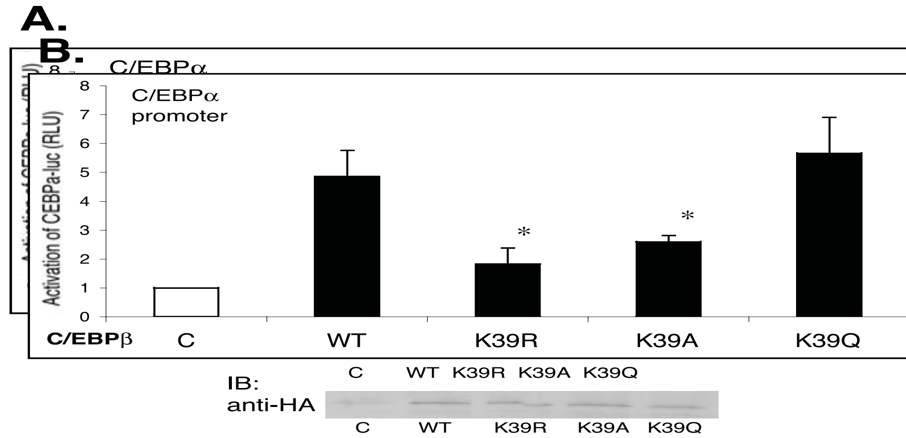


Fig 2.7. Mutations of K39 of C/EBPβ impair transcriptional activation of the C/EBPα promoter. A, C/EBPα-luc was co-expressed with C/EBPβ plasmids as in Fig. 6A and luciferase activity was measured (n=3 experiments). Activation of the C/EBPα promoter by K39R C/EBPβ is significantly lower ($p < 0.01$, asterisk) than by WT C/EBPβ. Transcriptional activation by WT C/EBPβ is significantly greater ($p < 0.01$) than control, and activation by R42A and K98R C/EBPβ is not significantly different from WT C/EBPβ. **B**, The plasmid for C/EBPα-luc was co-expressed with plasmids for C/EBPβ without (WT) or with mutations in K39 and luciferase activity was measured (n=3 experiments). Activation of the C/EBPα promoter is significantly lower ($p < 0.01$, asterisks) with K39R and K39A C/EBPβ than with WT C/EBPβ. Transcriptional activation of C/EBPα-luc by WT C/EBPβ is significantly greater ($p < 0.001$) than control. Activation by K39Q C/EBPβ is not significantly different from WT C/EBPβ.

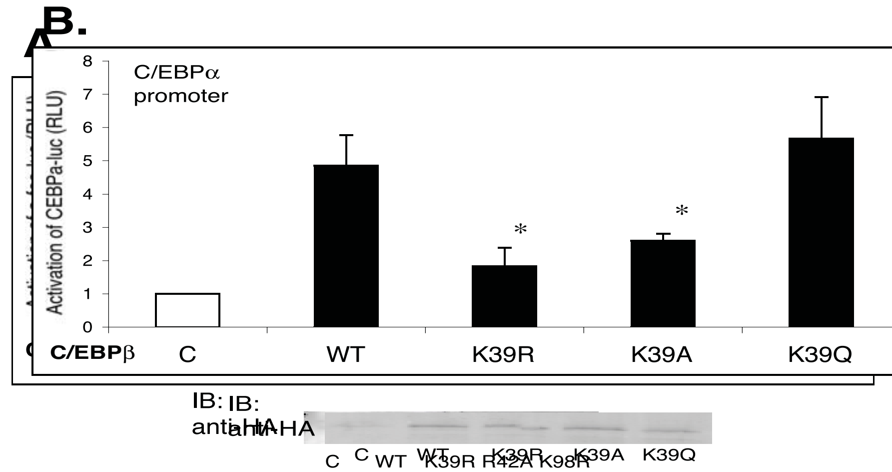


Fig 2.8. The integrity of K39 of C/EBPβ contributes to its ability to activate the *c-fos* promoter. **A**, The plasmid c-*fos*-luc was co-expressed with C/EBPβ plasmids with the indicated mutations at K39, R42, or K98, as in Fig. 6A and luciferase activity was measured (n=3 experiments). Activation of the c-*fos* promoter by K39R C/EBPβ is significantly lower ($p < 0.001$, asterisk) than by WT C/EBPβ. Transcriptional activation by WT C/EBPβ is significantly greater ($p < 0.001$) than control, and activation by R42A or K98R C/EBPβ is not significantly different from WT C/EBPβ. **B**, The plasmid for c-*fos*-luc was co-expressed with plasmids for C/EBPβ without (WT) or with the indicated mutations at K39, and luciferase activity was measured (n=3 experiments). Activation of c-*fos* is significantly lower ($p < 0.001$, asterisks) with K39R and K39A C/EBPβ than WT C/EBPβ. Transcriptional activation of the c-*fos* promoter by WT C/EBPβ is significantly greater ($p < 0.001$) than control, and activation by K39Q C/EBPβ is not significantly different from WT C/EBPβ.

acetylation functionally (3). Consistent with this, K39Q C/EBPβ increased activation via the consensus C/EBP site to the same extent as WT C/EBPβ (Fig 2.6B). Together, these findings indicate that acetylation at K39 contributes to the ability of C/EBPβ to mediate transcriptional activation via a consensus C/EBP site.

Mutation of K39 of C/EBPβ impairs its ability to activate the C/EBPα promoter

C/EBPβ activates the *C/EBPα* promoter as part of a transcription factor cascade during adipogenesis (25, 70, 71). The ability of WT and K39R C/EBPβ to activate the *C/EBPα* promoter was compared. WT C/EBPβ increased activation of the *C/EBPα* promoter more than six times above control (Fig 2.7A). Mutation K39R in C/EBPβ significantly reduced its ability to activate the *C/EBPα* promoter by more than 60%. In contrast, R42A and K98R C/EBPβ increased promoter activity to the same extent as WT C/EBPβ. These results indicate that the impairment in the ability of K39R C/EBPβ to activate the *C/EBPα* promoter is not secondary to general disruption of the transcriptional activation domain. Mutation K39A, like K39R, inhibited the ability of C/EBPβ to activate the *C/EBPα* promoter (Fig 2.7B). Interestingly, mutation of K39 to glutamine (Q), which mimics acetylation, increased *C/EBPα* promoter activation and was as effective as WT C/EBPβ. Together, these findings support the functional importance of acetylation of C/EBPβ at K39 in its transcriptional activation of *C/EBPα.*

Activation of the c-*fos* promoter depends on acetylation of C/EBPβ at K39

c-*fos* is a well-studied gene whose promoter can be activated by C/EBPβ (21, 46, 47, 72)**.** To test whether K39 of C/EBPβ contributes to its ability to activate c-*fos*, WT C/EBPβ or

C/EBPβ mutated at K39 was co-expressed with c-*fos*-luc (Fig 2.8A). WT C/EBPβ significantly increased promoter activity above control, as expected (21, 30). In contrast, mutation K39R in C/EBPβ completely blocked its ability to activate the c-*fos* promoter. C/EBPβ with other mutations in the transcriptional activation domain, R42A and K98R C/EBPβ, were comparable to WT C/EBPβ in their ability to activate the c-*fos* promoter, supporting specificity of K39 in the ability of C/EBPβ to activate c-*fos* transcription. Nevertheless, mutating K39 to A, which also impairs acetylation of C/EBPβ, decreased c-*fos* activation comparably to K39R (Fig. 8B), indicating a critical role for K39 in the function of C/EBPβ (50). K39Q C/EBPβ increased c-*fos* promoter activation to the same extent as WT C/EBPβ, consistent with glutamine mimicking acetylation. Together, these findings indicate that acetylation at K39 contributes to transcriptional activation of c-*fos*.

K39 of C/EBPβ contributes to GH-mediated activation of c-*fos*

C/EBPβ is a critical mediator of GH-stimulated c-*fos* expression, since knockdown of C/EBPβ prevents stimulation of c-*fos* RNA and promoter activation by GH (47). Because GH also increases the occupancy of both C/EBPβ and p300 on the c-*fos* promoter (47), it was reasoned that GH may regulate acetylation of C/EBPβ. GH was found to increase acetylation of C/EBPβ in 293T cells expressing WT C/EBPβ and GH receptor, as detected with the anti-Ac-K antibody that recognizes acetylation at K39 (Fig 2.9A). The increase suggests that acetylation of C/EBPβ in response to GH may play a role in its ability to activate transcription.

Since mutation of C/EBPβ at K39 alters the ability of C/EBPβ to activate c-*fos*, and since GH increases acetylation of C/EBPβ at K39, the role of K39 of C/EBPβ in GH-

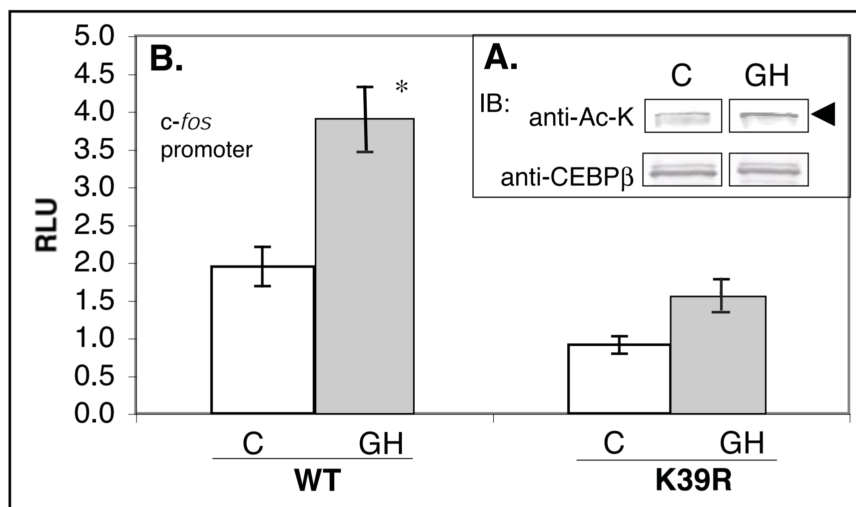


Fig 2.9. K39 of C/EBPβ contributes to GH-mediated activation of c-*fos*. A, WT HA-C/EBPβ and GHR were co-expressed in 293T cells, and cells were treated with GH (250 ng/ml) for 15 min. C/EBPβ was immunoprecipitated with anti-HA and used for immunoblotting with anti-Ac-K (upper panel) or anti-HA (lower panel). Arrowhead indicates acetylated C/EBPβ. Acetylation of C/EBPβ, calculated as Ac-K/ C/EBPβ (see methods), increased 2.2 times ± 0.37 control ($p < 0.04$) in response to GH in three experiments. **B**, Plasmids for WT HA-C/EBPβ or K39R C/EBPβ, and for GHR were co-expressed with c-*fos*-luc in CHO-GHR cells. 48 hr later, cells were treated with GH (500ng/ml) for 4 hr, lysed, and luciferase activity was measured. Bars (± SEM) represent luciferase activity in cells treated with vehicle control (C, open bars) or with GH (hatched bars) (n=4 experiments). Activation of c-*fos* in response to GH was significantly greater than control with WT C/EBPβ ($p < 0.001$, asterisk), but not with K39R C/EBPβ. In the absence of GH, basal activation of c-*fos* by K39R C/EBPβ was significantly decreased ($p < 0.05$) compared to WT C/EBPβ.

stimulated c-*fos* transcription was examined. GH stimulated the c-*fos* promoter in the presence of WT C/EBPβ (Fig 2.9B), as previously shown (21). However, in cells expressing K39R C/EBPβ, the response to GH was blunted and was not significant, even in the context of reduced basal promoter activity. The reduced response to GH when K39 in C/EBPβ is mutated suggests that GH increases acetylation of C/EBPβ, and also that acetylation of C/EBPβ at K39 contributes to activation of c-*fos* by GH. Not only is K39 a novel acetylation site on C/EBPβ that mediates activation of its gene targets, but its acetylation also appears to be regulated.

Different patterns of acetylatable lysines C/EBPβ differentially activate C/EBPβ-regulated promoters

The pattern of acetylatable lysines in C/EBPβ which mediate c-*fos*activation was found to be distinct from the patterns observed for the other promoters tested. For c-*fos*, mutation of C/EBPβ at K117 or K215/216 failed to impair activation when compared to WT C/EBPβ (Fig 2.10A), in contrast to the inhibition seen with K39R or K39A. Reinforcing the importance of K39, c-*fos* promoter activation was also reduced when C/EBPβ was mutated at K39 in combination with K117 and 215/216 (TM). These findings suggest that the integrity of K39 is a major contributor to the ability of C/EBPβ to activate the c-*fos* promoter.

Whether K39 is the only acetylatable lysine that contributes to transcriptional activation by C/EBPβ was tested by comparing other promoters to c-*fos*. Mutations K39R, K117R and TM (which contains K39R) C/EBPβ, but not K215/216R, decreased the ability of C/EBPβ to activate a C/EBP consensus site compared to WT C/EBPβ (Fig

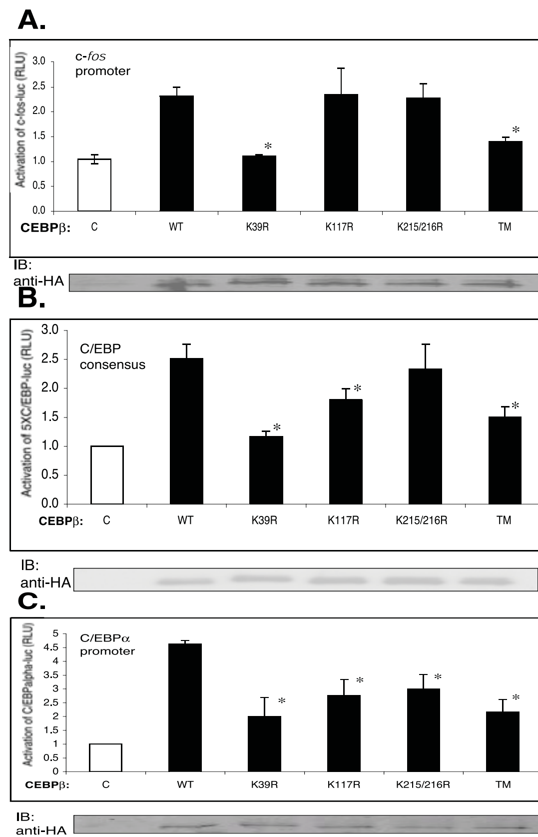
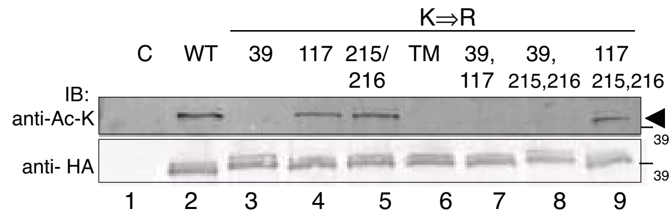
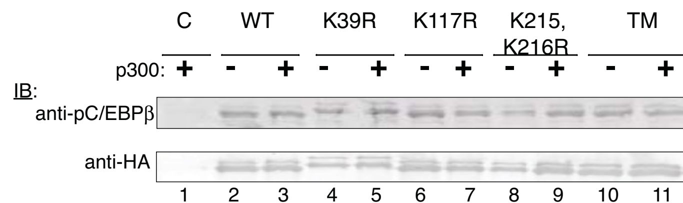


Fig 2.10. Different patterns of lysine mutations impair transcription on different C/EBPβ-responsive promoters. **A**, c-*fos* promoter: The plasmid for c-*fos*-luc was co-expressed with plasmids for C/EBPβ without (WT) or with mutant C/EBPβ alone or in combination (TM), and luciferase activity was measured (n=3 experiments). Cells were lysed 48 hr later and luciferase activity was measured (n= 3 independent experiments). Activation of c-*fos* by K39R and TM C/EBPβ is significantly lower ($p < 0.001$, asterisks) than WT C/EBPβ. Transcriptional activation of the c-*fos* promoter by WT C/EBPβ is significantly greater ($p < 0.001$) than control, and activation by K117R or K215/216R C/EBPβ is not significantly different from WT C/EBPβ. **B**, Consensus C/EBP site: The plasmid for 5X-C/EBP-luc was co-expressed with C/EBPβ plasmids as in Fig. 10A and luciferase activity was measured (n=5). Transcriptional activation by WT C/EBPβ is significantly ($p < 0.001$) greater than control. Activation by K39R ($P < 0.0001$), K117R ($p < 0.01$) and TM ($p < 0.001$) is significantly less (asterisks) than WT C/EBPβ. **C**, C/EBPα promoter: The plasmid for C/EBPα-luc was co-expressed with C/EBPβ plasmids as in Fig. 10A and luciferase activity was measured (n=3). Transcriptional activation by WT C/EBPβ is significantly ($p < 0.001$) greater than control. Activation of the C/EBPα promoter is significantly lower (asterisks) than WT C/EBPβ for K39R ($p < 0.001$), K117R ($p < 0.01$), K215/216R ($p < 0.05$), and TM ($p < 0.001$).

Supplemental Fig S2.1.



Supplemental Fig S2.2.



Supplemental Fig S2.1. Combinations of mutations of C/EBPβ that include K39R impair acetylation of C/EBPβ. Plasmids for HA-C/EBPβ without (WT) or with mutations (K⇒R) at indicated lysines, alone or in various combinations as indicated, were co-expressed with p300 in 293T cells. C/EBPβ was immunoprecipitated with anti-HA and used for immunoblotting with anti-Ac-K (upper panel) or anti-HA (lower panel). Similar results were obtained in at least three experiments. A decrease in acetylation was observed only when K39 of C/EBPβ was mutated, alone or in combination with other lysines.

Supplemental Fig S2.2. **Mutating candidate acetylation sites within C/EBPβ does not impair its phosphorylation at T188.** Plasmids for HA-C/EBPβ without (WT) or with mutations at indicated lysines were co-expressed without (lanes 2,4,6,8,10) or with p300 (3,5,7,9,11) in 293T cells. C/EBPβ was immunoprecipitated with anti-HA and used for immunoblotting with antibody specific for C/EBPβ phosphorylated at T188 (anti-pC/EBPβ, upper panel) or anti-HA (lower panel). Similar results were obtained in three experiments. Mutating candidate acetylation sites within C/EBPβ did not impair its phosphorylation at T188.

2.10B). Thus, for this C/EBP consensus site, acetylation at K117 as well as K39 contributes to activation by C/EBPβ. A different pattern was observed for the *C/EBPα* promoter (Fig 2.10C), on which mutation of each of the four lysines tested significantly decreased transcription activation. These findings emphasize that different patterns of acetylatable lysines within C/EBPβ are differentially required to activate C/EBPβ-regulated promoters.

Discussion

Lysine 39 is a novel acetylation site in C/EBPβ

These studies show that C/EBPβ is acetylated at K39 and that the ability of C/EBPβ to activate transcription is dependent on its integrity at K39. Acetylation of endogenous, as well as expressed, C/EBPβ was detected *in vivo*. Lysine 39 of C/EBPβ was initially identified as a candidate by its acetylation in C/EBPβ peptides incubated with p300 *in vitro*. Acetylation at this site by p300 and P/CAF in intact C/EBPβ was substantiated using an antibody that recognizes acetylated WT C/EBPβ but not C/EBPβ mutated at K39. Acetylation at K39 is of note because this lysine lies in the transcriptional activation domain of C/EBPβ (69).

The anti-Ac-K antibody appears to be specific for detecting acetylation of K39 in C/EBPβ, since mutation of K39 almost completely obliterated the anti-Ac-K signal, while mutation at other candidate (117, 215,216) and non-candidate (98) lysines did not interfere with detecting the signal. The anti-Ac-K antibody also detected a decrease in acetylation when K39 was mutated in combination with K117, K215, and K216 (TM).

Several other anti-Ac-K antibodies were not effective (data not shown). The present work using the anti-Ac-K antibody and mutated C/EBPβ strongly supports that K39 of C/EBPβ is acetylated.

C/EBPβ is acetylated at multiple lysines

While acetylation of C/EBPβ has been reported, (44, 45), acetylation of K39 as a specific acetylation site important for transcriptional activation is novel. Nevertheless, K39 is only one of multiple acetylatable lysines in C/EBPβ. A previous report that K215 and K216 are acetylated is supported by the present observation that a C/EBPβ peptide corresponding to K215/216 was acetylated when incubated with p300 *in vitro*. Deacetylation of C/EBPβ at these residues allowed transcriptional activation of the Id-1 gene, mediated by recruitment of HDAC1 by Stat5 in response to IL-3 in Ba/F3 cells (45). In the present study, mutation of K215/216 did not alter transcriptional activation of c-*fos,* suggesting different roles for these lysines for different genes such as Id-1 and c-*fos*. The acetylation site at K39 differs from the acetylation motif containing two adjacent lysines at K215/216 of C/EBPβ, where acetylation inhibits DNA binding on the Id-1 promoter (45). For the single lysine at K39, which lies in the transcriptional activation domain, acetylation appears to be activating rather than inhibitory, and contributes to activation of c-*fos* and C/EBPα, physiological targets of C/EBPβ.

Another novel acetylation site was identified at K117 of C/EBPβ. K117 was detected with C/EBPβ peptides incubated with p300 *in vitro*, but mutation of K117 in intact C/EBPβ did not impair the ability of the anti-Ac-K antibody to detect acetylated C/EBPβ, even when several antibodies against acetylated lysine were tested (data not

shown). However, MALDI-TOF analysis of purified HIS-C/EBPβ incubated with p300 *in vitro* identified acetylation of C/EBPβ at K117 (data not shown). It is also of interest that mutation of K117, unlike K39R, did not impair transcriptional activation of the c-*fos* promoter. Further analysis of acetylation of K117 of C/EBPβ will be informative. Whether additional acetylation sites exist within the C-terminal region of C/EBPβ remains to be determined. Attempts to distinguish relative acetylation at candidate lysines using GST-C/EBPβ without and with lysine mutations were inconsistent. Thus, relative acetylation at K39, K117 and K215/216 cannot be resolved by this approach.

P/CAF, like p300, increased acetylation of intact C/EBPβ, although P/CAF did not acetylate C/EBPβ peptides. Acetylation of C/EBPβ by P/CAF may depend on sequences surrounding acetylated lysines of C/EBPβ, since protein modifications on one site of a molecule can be dependent on distal or nearby sequences within the same molecule. For example, glycosylation of the Wnt family member, Wingless, requires the integrity of surrounding sequences its N terminus (73). Thus, P/CAF may acetylate C/EBPβ in the context of an intact C/EBPβ sequence, but not under conditions used with the C/EBPβ peptides.

Acetylation and phosphorylation of nuclear factors can be interdependent (10). For example, p53 phosphorylation activates its acetylation, most likely by increasing association of p53 with p300 (4, 74). Acetylation of Foxo1 also increases its phosphorylation at S253 and decreases its ability to bind DNA (75). Because there are multiple phosphorylation sites in C/EBPβ, it is tempting to speculate that phosphorylation and acetylation of C/EBPβ may influence each other. However, there was no alteration in the acetylation of C/EBPβ at K39 when phosphorylation sites S184 or T188 in

C/EBPβ were mutated. Conversely, there was no alteration in the phosphorylation of C/EBPβ at T188 when K39, K117 or K215/216 was mutated. Thus, the present studies do not support an interdependent relationship between phosphorylation of C/EBPβ at two regulated phosphorylation sites, S184 and T188, and acetylation of C/EBPβ at K39, under conditions tested. This does not preclude that acetylation at other lysines in C/EBPβ may be dependent on its phosphorylation at T188 or other phosphorylation sites.

Since lysines are subject to other modifications such as sumoylation, ubiquitination, and methylation (76), it will be important to consider whether other modifications occur on K39 or other lysines in C/EBPβ. Preliminary data suggest that K39 of murine C/EBPβ is not sumoylated (Piwien-Pilipuk, G, Subramanian, L, unpublished results), although K133 of C/EBPβ (corresponds to K173 of human C/EBPβ) is reported to be sumoylated (77, 78).

Mutation of K39 of C/EBPβ alters transcriptional activation of its target genes

Mutation of C/EBPβ at K39 consistently impaired activation of the promoters of C/EBPβ target genes, including those for physiological targets c-*fos* and C/EBPα, as well as a consensus C/EBP site. K39 lies in the transcription activation domain of C/EBPβ, but other mutations in the transcriptional activation domain that were tested (R42A and K98R) did not impair the ability of C/EBPβ to activate target genes. This is also consistent with lack of acetylation of a peptide containing K98 (Fig 2.4A). Together, these observations indicate that the impaired transcription with K39R C/EBPβ does not simply disable the transcription activation domain. Additionally, mutation of K39 to

glutamine, which functionally mimics acetylation (3), activated the three target gene promoters tested comparably to WT C/EBPβ, strengthening the likelihood that acetylation at K39 of C/EBPβ is an event contributing to transcriptional activation by C/EBPβ. K39A, like K39R, both disrupted acetylation and decreased activation by C/EBPβ of c-*fos*, C/EBPα, and a consensus C/EBP site. Since the N-terminus of C/EBPβ has been reported to interact with p300 (40), impaired c-*fos* promoter activation by K39R and K39A C/EBPβ may reflect a role for the acetylation of C/EBPβ at the N-terminus in its recruitment of p300 (40), as reported for acetylation of other transcription factors such as p53 and GATA-2 (10, 79). p300 is recruited to the c-*fos* promoter and is present in a complex with C/EBPβ (47).

GH-induced acetylation of C/EBPβ contributes to transcription of c-*fos*

Since K39 appears to be exclusively required for c-*fos* activation among the four lysines tested, regulation of acetylation of K39 and of c-*fos* by GH was examined. This study demonstrates that GH can increase the acetylation of C/EBPβ as detected by anti-Ac-K, which recognizes acetylation at K39. Further, since the ability of GH to activate c-*fos* was impaired by mutation K39R C/EBPβ, acetylation of K39 may contribute to c-*fos* activation by GH. Acetylation of C/EBPβ may thus be a factor influencing the function of this transcription factor in response to GH, as phosphorylation has been shown to do (30).

Patterns of acetyatable lysines in C/EBPβ differ for activation of different gene targets

It is of interest that different patterns of mutation of acetylatable lysines in C/EBPβ interfered with transcriptional activation for different promoters responsive to C/EBPβ, suggesting context-dependent regulation of its acetylation. On the c-*fos* promoter, K39 appears to be the single acetylatable lysine of C/EBPβ among those tested whose integrity is required for transcriptional activation. On the consensus C/EBP site, both K39 and K117 must be intact, and for C/EBPα activation, the integrity of all four acetylatable lysines tested appears to be required for transcription. The functional importance of each acetylation site does not appear to parallel the extent of acetylation at each lysine. Experiments with truncations of C/EBPβ suggest that relative acetylation of C/EBPβ exhibited by K39 represents at most 50% of the acetylation among the four lysines tested. However, for c-*fos,* acetylation of K39 alone appears to be sufficient for transcription. For the Id-1 promoter, it is not clear whether K215/216 in C/EBPβ are the only residues whose regulation alters transcriptional activation. Overall, the relative degree of acetylation does not appear to be the determinant of function. Rather, differences in the patterns of acetylation among acetylatable lysines in C/EBPβ appear to confer varying effectiveness for transcriptional activation on different promoters.

Acetylation, like phosphorylation (80), may serve as a regulated molecular switch for transcription (10). The regulation of acetylation, as well as phosphorylation, of C/EBPβ introduces greater opportunity for specificity among its range of functions. This is of note with a transcription factor such as C/EBPβ which modulates genes involved in

a wide variety of processes, including immune responses, liver regeneration and adipocyte differentiation (12-14, 19, 25, 81). Overall understanding of the functional importance of acetylation of C/EBPβ can provide insight into regulatory networks for a variety of cellular functions mediated by changes in gene transcription

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