O-Linked N-Acetylglucosamine Modification on CCAAT Enhancer-binding Protein β

ROLE DURING ADIPOCYTE DIFFERENTIATION*

Received for publication, April 8, 2009, and in revised form, May 8, 2009 Published, JBC Papers in Press, May 28, 2009, DOI 10.1074/jbc.M109.005678

Xī Lì§, Henrīk Molīna§, Haitiān Huāng§§, You-you Zhāng§§, Mei Liú, Shu-wēn Qiān§§, Chad Slawson‡, Wagner B. Diās‡, Akhišeḷ Dānede¶, Gerald W. Hart‡, M. Daniel Lane¶, and Qi-Qún Tāng¶§

From the §Key Laboratory of Molecular Medicine, Ministry of Education, and Department of Biochemistry and Molecular Biology, Fudan University Shanghai Medical College, Shanghai 200032, China, the ¶Institute of Stem Cell and Regenerative Medicine, Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China, and the *Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21218

CCAAT enhancer-binding protein (C/EBP)β is a basic leucine zipper transcription factor family member, and can be phosphorylated, acetylated, and sumoylated. C/EBPβ undergoes sequential phosphorylation during 3T3-L1 adipocyte differentiation. Phosphorylation on Thr188 by MAPK or cyclin A/cdk2 primes the phosphorylations on Ser184/Thr179 by GSK3β, and these phosphorylations are required for the acquisition of DNA binding activity of C/EBPβ. Here we show that C/EBPβ is modified by O-GlcNAc, a dynamic single sugar modification found on nucleocytoplasmic proteins. The GlcNAcylation sites are Ser180 and Ser181, which are in the regulation domain and are very close to the phosphorylation sites (Thr188, Ser184, and Thr179) required for the gain of DNA binding activity. Both in vitro and ex vivo experiments demonstrate that GlcNAcylation on Ser180 and Ser181 prevents phosphorylation on Thr188, Ser184, and Thr179, as indicated by the decreased relative phosphorylation and DNA binding activity of C/EBPβ delayed the adipocyte differentiation program. Mutation of both Ser180 and Ser181 to Ala significantly increase the transcriptional activity of C/EBPβ. These data suggest that GlcNAcylation regulates both the phosphorylation and DNA binding activity of C/EBPβ.

*This work was supported by National Key Basic Research Project Grant 2006CB943704, National Natural Science Foundation for Distinguished Scholars Grant 30625015, Program for Outstanding Medical Academic Leader Grant B-L06032, Shanghai Key Science and Technology Research Project 08dj1400603 (to Q.-Q. T.); by National Natural Science Foundation Grant 30700121 and Shanghai Rising Star Program 08QQA14012 (to X. L.), and by National Institutes of Health Grants DK61671 and HD13563 (to G. W. H.). This work was also supported in part by Shanghai Leading Scholars Grant 30625015, Program for Outstanding Medical Academic Leaders Grant 30700121 and Shanghai Rising Star Program 08QA14012 (to X. L.), and Shanghai Leading Scholars Grant 30625015, Program for Outstanding Medical Academic Leaders Grant 30700121 and Shanghai Rising Star Program 08QA14012 (to X. L.), and these phosphorylations are required for the gain of DNA binding activity of C/EBPβ. C/EBPβ contains a C-terminal basic region leucine zipper DNA-binding domain, a dimerization domain, and an N-terminal transactivation domain together with a regulation domain (RD), which contains multiple putative modification sites in the middle. C/EBPβ assumes a tightly folded conformation in which the activation domain (N-terminal) and DNA-binding domain (C-terminal) are obscured by interaction with the RD. Appropriate modifications disrupt the RD interaction rendering the binding domain accessible for DNA binding and thereby facilitating transactivation. Three phosphorylation sites (Thr188, Ser184, and Thr179) have been identified in this RD region of C/EBPβ during 3T3-L1 adipocyte differentiation (14). Both ex vivo and in vitro experiments demonstrate that phosphorylation on Thr188 (first by MAPK during G1 phase and late by CyclinA/cdk2 (15)) primes C/EBPβ for the phosphorylation on Ser184 or Thr179 by GSK3β, and these phosphorylations are required for the gain of DNA binding activity of C/EBPβ (14, 16).

O-Linked β-N-acetylglucosamine modification (GlcNAcylation) (17) is a novel glycosylation in which the monosaccharide β-N-acetylglucosamine (GlcNAc) attaches to serine/threonine residues via an O-linked glycosidic bond and is found mostly within the cytoplasm or nucleoplasm (18). GlcNAcylation regulates several important cellular processes, such as signal transduction, protein expression, degradation, and trafficking (19). This post-translational modification is regulated by two highly conserved enzymes: OGT and O-GlcNAcase (β-N-acetylglucosaminidase), whereas OGT catalyzes the attachment and O-GlcNAcase catalyzes the removal of O-GlcNAc.
GlcNAcylation of C/EBPβ

N-acetylglucosamine (GlcNAc) is dynamically regulated in a manner similar to phosphorylation. O-GlcNAc and O-phosphate site-mapping studies suggest that there are several different types of dynamic interplay between O-GlcNAc and O-phosphate: 1) competitive occupancy at the same site; 2) competitive and alternative occupancy at proximal sites; 3) a complex interplay whereby some O-phosphate attachment sites on a given protein are the same as some O-GlcNAc sites, whereas others are adjacent to, or even distant from each other; and 4) phosphorylation regulates the O-GlcNAc cycling enzymes and O-GlcNAc regulates kinases (19). The C-terminal domain of RNA polymerase II is extensively GlcNAcylated (22), and almost all RNA polymerase II transcription factors are modified by the sugar. GlcNAcylation can either suppress or enhance transcription, depending on the promoter involved, on the cell system, and on associations with different co-factors (23, 24).

C/EBPβ can be phosphorylated (25), acetylated (26), and sumoylated (27). In this study, we find that C/EBPβ is GlcNAcylated on Ser180 and Ser181, which is in the RD region and adjacent to the phosphorylation sites (Thr188, Ser184, and Thr179) required for the gain of DNA binding activity. GlcNAcylation on Ser180 and Ser181 prevents the phosphorylation on Thr188, Ser184, and Thr179. Mutations of Ser180 and Ser181 to alanine residues significantly increase the transcriptional activity of C/EBPβ. This suggests that GlcNAcylation regulates both the phosphorylation and DNA binding activity of C/EBPβ, and GlcNAcylation and phosphorylation regulate the transcriptional activities of C/EBPβ in a competitive way and by alternative occupancy at adjacent sites.

EXPERIMENTAL PROCEDURES

Cell Culture, Induction of Differentiation, and Transfection—3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (v/v) calf serum as described (30). To induce differentiation, 2-day postconfluent preadipocytes (designated day 0) were fed DMEM containing 10% (v/v) fetal bovine serum, 1 μg/ml insulin, 200 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine until day 2. The cells were then fed DMEM supplemented with 10% fetal bovine serum and 1 μg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% fetal bovine serum. To test the effect of GlcNAcylation on the C/EBPβ phosphorylation and subsequently adipocyte differentiation, the post-confluent 3T3-L1 preadipocytes were treated with O-GlcNAcase inhibitor, GlcNAc thialzoline (GT; Johns Hopkins Medical Chemical Core) 1 h before and at the time exposed to the differentiation inducers. At the various times indicated, whole cell lysates were prepared and subjected to SDS-PAGE and detected with antibodies against total C/EBPβ, phospho-C/EBPβ, PPARγ, and 422a/p2 antibodies; relative protein levels were quantitated and plotted.

A C/EBPα promoter report construct was made by cloning 2044 bp of the mouse C/EBPα promoter into luciferase vector pGL3.0. C/EBPβ expression vectors were cloned by polymerase chain reaction (PCR) of wild type mouse C/EBPβ cDNA or mutants (Ser180 → Ala and/or Ser181 → Ala) into pcDNA3.1, and the mutants were generated by using Stratagene Quick change site-directed mutagenesis kit. After reaching 90% confluence, 3T3-L1 cells were transfected with equal amount of report construct and/or C/EBPβ expression vectors, a thymidine kinase Renilla luciferase construct (Promega) was co-transfected and used as transfection efficiency control. Two days after transfection, the cells were induced as above, and 36 h after induction cells were lysed, and luciferase activity was determined by dual luciferase assay kit (Promega).

Western Blotting—Equal amounts of protein were subjected to SDS-PAGE and immunoblotted with antibodies against C/EBPβ, Thr(P)-188-C/EBPβ (Cell Signaling Technology, Beverly, MA), CTD110.6, 422a/p2, and PPARγ (Santa Cruz, Santa Cruz, CA). Antibody against C/EBPβ and 422a/p2 were prepared in this laboratory. CTD110.6 was used for O-GlcNAc detection (from Dr. G. W. Hart’s lab).

Preparation of Recombinant C/EBPβ-LAP and C/EBPβ-LIP Protein—The cDNA encoding full-length C/EBPβ-LAP protein and the C-terminal 152–289 amino acids of C/EBPβ-LIP were cloned into the downstream of GST gene in pGEX-6P (Amersham Biosciences) and transformed into Escherichia coli strain BL21 (DE3) pLysS (Novagen). A single colony was propagated overnight in 3 ml of LB medium containing ampicillin and chloramphenicol, and then diluted (1:100) into 500 ml of fresh LB medium the next day and cultured until an A600nm of 0.6–0.7 was reached. Expression of the fusion proteins was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside for 3 h, and the cells were harvested and resuspended in 25 ml of phosphate-buffered saline containing 1% Triton X-100. After lysis by one cycle of freezing-thawing, the cell suspension was treated with DNase I and RNase A and then incubated on ice for 10 min in the presence of 0.5 mM NaCl and 5 mM dithiothreitol to dissociate DNA-protein complex. After centrifugation at 12,000 rpm for 10 min, 250–500 μl of bed volume of GSH-Sepharose (GE Healthcare) was added to the supernatant and mixed overnight in a cold room. The beads were washed three times with phosphate-buffered saline, washed once with PreScission cleavage buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol), and then treated with 80 units of PreScission Protease (GE Healthcare) in 960 μl of buffer. The cleaved recombinant C/EBPβ protein was further purified by CM-Sepharose column (GE Healthcare), and soluble, highly purified recombinant protein was eluted at 250–300 mM of NaCl. The protein concentration was determined by Bradford assay, and the purity of protein was verified by SDS-PAGE.

In Vitro Phosphorylation and Mass Analysis of C/EBPβ—Two micrograms of recombinant C/EBPβ-LAP were incubated with activated MAPK (Calbiochem) and GSK3β (Upstate) in buffer containing 50 mM HEPES (pH 7.0), 10 mM MgCl2, 1 mM dithiothreitol, and 100 mM cold ATP or 20 μCi of [γ-32P]ATP (1 Ci = 37 GBq) at 30 °C for 30 min. The phosphorylation was detected with Thr(P)-188 phospho-specific antibody or by autoradiography after the protein was separated by SDS-PAGE. For identification of phosphorylation sites on C/EBPβ, similar experiments were performed with 0.5 mM ATP. The protein band containing C/EBPβ was excised from the gel, digested with trypsin, and analyzed by nano liquid chromatography-tandem mass spectrometry as previously described (26).
GlcNAcylation of C/EBPβ

To investigate the effect of O-GlcNAcylation on the O-phosphorylation of C/EBPβ, GST-C/EBPβ-LAP fusion protein (which bound to the GSH-Sepharose) was used as substrate, GST-C/EBPβ-LAP was GlcNAcylated by OGT first and then used for substrate for *in vitro* phosphorylation as described above, at times indicated reactions were stopped by adding loading buffer and separated by SDS-PAGE, the phosphorylation was detected by Western blotting with Thr(188) phospho-specific antibody or autoradiography (detecting of all phosphorylation).

In Vitro O-GlcNAcylation and Identification of O-GlcNAc Sites of C/EBPβ—Two μg of recombinant C/EBPβ was incubated with 1 μg of recombinant OGT in buffer containing 50 mM sodium cacodylate (pH 6.5), 1 mg/ml bovine serum albumin, 35 mM NaF, 2 μM 2-acetamido-2-deoxy-D-gluconohydroximolactone, 2.5 mM AMP, and 1 mM UDP-GlcNAc (or 1 Ci of [3H]UDP-GlcNAc) at 25 °C for 60 min. The GlcNAcylation was detected by Western blotting with O-GlcNAc-specific antibody CTD110.6 or autoradiography after the protein was separated by SDS-PAGE. For identification of O-GlcNAcylated sites on C/EBPβ, the protein band containing C/EBPβ was excised from the gel and destained. The protein bands were digested overnight with trypsin (Promega, Madison, WI), and then the peptides were extracted and lyophilized. For some samples, the generated tryptic peptides were subjected to a second digestion using chymotrypsin. In those cases the tryptic peptides were redissolved in 16 μl of 100 mM ammonium bicarbonate containing 10 ng/μl of high purity bovine pancreas chymotrypsin (Calbiochem, La Jolla, CA). The second part of this sequential digestion was also allowed to proceed overnight. All of the digestions were stopped with acetic acid. Lyophilized peptides were redissolved in 20 μl of 3% acetonitrile, 0.1% formic acid prior to liquid chromatography–tandem mass spectrometry analysis.

Mass spectrometry analyses were performed using a ion trap fitted with a chip cube LC system and electron transfer dissociation (ETD) capability (Agilent Technologies, Santa Clara, CA), as previously described. The ion trap was operated in 1) MS followed by alternating CID and ETD, 2) MS only; or 3) CID only on selected precursors.

Detection of GlcNAcylation on Endogenous C/EBPβ—3T3-L1 cells were differentiated as above and harvested at the indicated time. C/EBPβ was immunoprecipitated, washed with lysis buffer, and washed twice with reaction buffer containing 20 mM Hepes, pH 7.9, 50 mM NaCl, 1 μM 2-acetamido-2-deoxy-D-gluconohydroximolactone, and 5 mM MnCl2 with protease and phosphatase inhibitors. Next, 2 μl of Gal-T1 Y289L (Invitrogen) and 2 μl of 0.5 μM UDP-GalNAz (Invitrogen) were added to a reaction volume of 20 μl. The reaction was performed overnight at 4 °C. The beads were washed twice with reaction buffer to remove excess UDP-GalNAz. The sample were then reacted with biotin-alkyne (Invitrogen) according to the manufacturer’s instructions. The enzymatic reactions were eluted with Laemmli buffer for immunoblot analysis.

Chromatin Immunoprecipitation (ChIP) Analysis—ChIP analysis was performed essentially as described in the protocol of ChIP assay kit (Upstate Biotechnology). Briefly, 3T3-L1 preadipocytes were maintained and induced to differentiate as above. O-GlcNAcase inhibitor (GT; Johns Hopkins Medical Chemical Core) was added 1 h before and added again at the time of induction. At various times indicated, the cells were cross-linked with 1% formaldehyde in phosphate-buffered saline buffer. The cross-linked cells were harvested, lysed in SDS lysis buffer, and sonicated. After incubation with salmon sperm DNA–protein A overnight at 4 °C, the DNA–protein complexes in the supernatant were immunoprecipitated with antibodies against C/EBPβ, and the immune complexes were recovered by adding protein A-agarose. After series washing, DNA was extracted with phenol/chloroform, precipitated, redissolved, and used as a template for PCR with the following primers. The primers used to amplify the DNA fragments containing the C/EBP-binding site in C/EBPa promoter: TCCCTAGTGTTGGCTGGAAG and CAGTAGGATGTTGCCGGAAG and CAGTAGGATGTTGCCCTGCTG.

RESULTS

GlcNAcylation of Ser180 and Ser181 in the RD of C/EBPβ has two isoforms (liver activation protein (LAP) and liver inhibitory protein (LIP); Fig. 1A) both of which are derived from the same mRNA by using different translation start codons (28). These two isoforms share the same DNA-binding domain and leucine zipper domain in their C terminus and the RD in the upstream of DNA-binding domain, whereas LAP has a transactivation domain in the N terminus and LIP does not. After treatment with OGT and unlabeled UDP-GlcNAc, both recombinant LAP and LIP are GlcNAcylated as evidenced by Western blotting with O-GlcNAc specific antibody CTD110.6 (Fig. 1, B and C, middle panels), and incubation with OGT and [3H]UDP-GlcNAc labels both LAP and LIP well, as indicated by autoradiography (Fig. 1, B and C, bottom panels). These data indicate that C/EBPβ can be GlcNAcylated and that the residue or residues very likely are located in the DNA-binding and/or regulation domain of C/EBPβ, which is shared by both LAP and LIP.

By combining ETD and CID mass spectrometry with liquid chromatography, we were able to map GlcNAcylation sites as well as O-linked phosphorylations of C/EBPβ. We used CID to identify peptides showing a characteristic fragment pattern for O-GlcNAc-modified peptides and ETD to pin-point the modified residue(s) of these peptides. After treatment with OGT and cold UDP-GlcNAc, C/EBPβ (LAP) were purified by SDS-PAGE. The C/EBPβ–LAP protein band was excised and trypsinized. A triply charged tryptic peptide was identified, and this peptide was found to be H-GlcNAc-Glu-Pro, which indicated that C/EBPβ has an O-GlcNAcylated residue at position 180, as indicated by Mass spectrometry analysis (Fig. 1A). For the nonrecombinant LAP (LIP), C/EBPβ was GlcNAcylated by OGT first and then treated with Gal-T1 Y289L as described. Western blotting showed that the LAP (LIP) was GlcNAcylated at Ser181, as indicated by the upper band (Fig. 1B). 

![Figure 1](https://example.com/figure1.png)
AYLGQATPSGGSSPGLSTSSSSSPGTPSPADAK was found to be a O-GlcNAc-modified peptide. However, because of the high number of potential modified residues (14 residues) neither CID nor ETD allowed us to accurately pin-point the modified residue. By subjecting the already trypsinized sample to a second digestion, this time using chymotrypsin, we were able to create smaller peptides carrying five and nine potential modified residues, respectively, thereby greatly simplifying our task. Peptides with the sequence STSSSSSPPGTPSPADAK showed the GlcNAcylation fragmentation characteristics and were selected for ETD-based GlcNAcylation site mapping. The same sequence was found to carry either one or two O-GlcNAc modifications. When carrying one O-GlcNAc, the modified residue was assigned as Ser\textsuperscript{180}. When carrying two O-GlcNAc modifications, the modified residues were pin-pointed as Ser\textsuperscript{180} and Ser\textsuperscript{181}. Fig. 2 shows the ETD spectra of the modified peptides. Fig. 2A shows the unmodified peptide, whereas the spectra of peptides carrying one and two O-GlcNAc units are shown in B and C.

GlcNAcylation Prevents the Phosphorylation of C/EBPβ—GlcNAcylation on Ser\textsuperscript{180} and Ser\textsuperscript{181} are in the RD region of C/EBPβ and adjacent to three phosphorylation sites (Thr\textsuperscript{179}, Ser\textsuperscript{184}, and Thr\textsuperscript{188}) we identified before (14). To confirm and extend our previous study, C/EBPβ-LAP was phosphorylated with MAPK and GSK3β, and phosphorylations on Thr\textsuperscript{188}, Ser\textsuperscript{184} (Fig. 2, D and E), and Thr\textsuperscript{179} (data not shown) were confirmed. Because the phosphorylations on Thr\textsuperscript{188}, Ser\textsuperscript{184}, and Thr\textsuperscript{179} are required for C/EBPβ to gain the DNA binding activity, the question was raised as to whether GlcNAcylation will affect the DNA binding activity. Recombinant C/EBPβ-LAP was phosphorylated with MAPK and GSK3β (or GlcNAcylated with OGT), and the phosphorylation was confirmed by Western blotting with Thr(P)\textsuperscript{188} phospho-specific antibody (Fig. 3B) and mass spectrometry analysis (similar to Fig. 2, D and E, and data not shown). GlcNAcylation was confirmed by Western blotting with O-GlcNAc specific antibody CTD110.6 (Fig. 3C). Electrophoretic mobility shift analysis was performed to compare the DNA binding activity; consistent with our previous findings (14), unmodified C/EBPβ-LAP had little or no DNA binding activity, whereas the DNA binding activity dramatically increased after phosphorylation by MAPK and GSK3β (Fig. 3D). GlcNAcylated C/EBPβ-LAP did not bind to the C/EBP consensus sequence similar to unmodified C/EBPβ-LAP (Fig. 3D).
Mutations on Ser180 and Ser181 Significantly Increase the Transphosphorylations; data not shown). The phosphorylation was confirmed by Western blotting with phospho-specific antibody (B). GlcNAcylation was confirmed by Western blotting with O-GlcNAc specific antibody CTD110.6 (C). DNA binding activity was detected by electrophoretic mobility shift analysis and compared (D). Modifications on C/EBPβ-LAP are illustrated (E). P-LAP, phosphorylated LAP; G-LAP, GlcNAcylated LAP.

**FIGURE 4.** GlcNAcylation prevents the phosphorylation of C/EBPβ in vitro. GST-C/EBPβ-LAP was first GlcNAcylated by incubation with OGT and then used as the substrate for further phosphorylation. At various time points the reactions were stopped, and the protein was separated by SDS-PAGE. The GlcNAcylated C/EBPβ was detected by Western blotting with CTD110.6 antibody (top panel). The GlcNAcylated C/EBPβ detected by Western blotting with CTD110.6 antibody (middle panel), and phosphorylated protein was detected by phospho-specific antibody (B). GlcNAcylation was confirmed by Western blotting with O-GlcNAc specific antibody CTD110.6 (C). DNA binding activity was detected by electrophoretic mobility shift analysis and compared (D). Modifications on C/EBPβ-LAP are illustrated (E). P-LAP, phosphorylated LAP; G-LAP, GlcNAcylated LAP.

GlcNAcylation (Ser160 and Ser181) and phosphorylation (Thr179, Ser184, and Thr188) do not occur on the same sites but occur very close to each other; next we asked whether these two different post-translational modifications could influence each other. To test this, GST-fused C/EBPβ-LAP was first GlcNAcylated and then used as the in vitro substrate for MAPK and GSK3β. We found that phosphorylation was significantly decreased and delayed as indicated by Western blotting (detecting Thr(P)188) (Fig. 4) and autoradiography (detecting all phosphorylations; data not shown).

GlcNAcylation Modulates the DNA Binding Activity, and Mutations on Ser180 and Ser181 Significantly Increase the Transcriptional Activity of C/EBPβ—A chemoenzymatic approach (29) was used to confirm GlcNAcylation on the endogenous C/EBPβ. Growth-arrested 3T3-L1 preadipocytes were induced to differentiation, and endogenous C/EBPβ was purified with immunoaffinity column (14), labeled by using a mutant galactosyltransferase GalT1 Y289L and an azide derivative of UDP-Gal (UDP-GalNAz) as donor substrate. The resulting azide-labeled C/EBPβ was chemically tagged with biotin-alkyne, and GlcNAcylation was detected by Western blotting with streptavidin-horseradish peroxidase. We found that C/EBPβ-LAP was strongly labeled with biotin, confirming the presence of O-GlcNAc in this protein (Fig. 5). To further test whether GlcNAcylation prevents phosphorylation of C/EBPβ in vivo, the GlcNAcylation of whole cell proteins was elevated, and the effects on the phosphorylation and DNA binding activity were investigated. After treatment with O-GlcNAcase inhibitor (GlcNAc-Thiazoline, GT), the GlcNAcylation of total cell proteins was significantly increased as detected by Western blotting with O-GlcNAc specific antibody CTD110.6 (Fig. 6C), we found that whole C/EBPβ protein level is increased (Fig. 6A) while relative phospho-C/EBPβ protein is decreased (Fig. 6, B and E), which indicates the ratio of phosphorylated C/EBPβ to total C/EBPβ is decreased (Fig. 6E). The ratio change was not due to the change in MAPK activity, which is responsible for the phosphorylation, and the phosphorylation and activation of MAPK indicated by p-MAPK, since p-MAPK was not affected by the O-GlcNAcase inhibitor (GT) (Fig. 6f).

ChIP analysis was performed to test whether O-GlcNAcase inhibitor (GT) treatment also affected the DNA binding activity of C/EBPβ after the phospho-C/EBPβ was decreased. Postconfluent 3T3-L1 preadipocytes were treated with/out GT and 24 h later cells were cross-linked, and ChIP analysis was performed on the DNA binding activity of C/EBPβ on the C/EBPα promoter. We found that the binding of C/EBPβ to the C/EBP element in C/EBPα promoter was significantly decreased (Fig. 7A) after relative phospho-C/EBPβ was decreased, and treatment with the O-GlcNAcase inhibitor (GT) delays the 3T3-L1 adipocyte differentiation program as evidenced by decreasing the expression of PPARγ and 422/aP2 ~20–30% after 48 and 72 h of MDI induction; however, the expression catches up at 96 h after MDI induction (Fig. 7, C and D).

Ser180 and Ser181 were also mutated to further investigate the function role of GlcNAcylation on C/EBPβ. We found that C/EBPβ can significantly transactivate C/EBPα promoter reporter, but neither mutant of S180A nor mutant of S181A alone had any effect. However, mutations on both Ser180 and Ser181 dramatically increased the transactivation of C/EBPβ (Fig. 7B).

**FIGURE 5.** C/EBPβ is GlcNAcylated In vivo. Post-confluent 3T3-L1 preadipocytes were induced to differentiation as described, and nuclear extracts were prepared at 4h and 24h after induction. C/EBPβ was successfully immunoprecipitated as indicated by Western blotting with specific antibody (A), and GlcNAcylated C/EBPβ was detected as described (B).
DISCUSSION

We have used tandem mass spectrometry to identify the O-GlcNAc residues on C/EBPβ. Although this approach in most cases is very sensitive and accurate, O-GlcNAc-modified residues can be challenging to pinpoint. First, enrichment of peptides carrying this modification is far from trivial, and second the O-GlcNAc modification is very labile when subjected to CID, making it difficult to accurately assign the modified residue(s) (31). To address the latter point, we decided to combine ETD and CID with liquid chromatography to map GlcNAcylation sites as well as O-linked phosphorylations of C/EBPβ. This newer fragmentation technique has proven to be an excellent choice to study so-called labile modifications exemplified by O-linked phospho-tyrosines and O-GlcNAc (24). We provide clear mass spectrometry data to show that C/EBPβ is GlcNAcylated on Ser 180 and Ser 181 by OGT and phosphorylated on Thr188/Ser184/Thr179 by MAPK and GSK3β (Fig. 2).

Increasing evidence indicates that most RNA polymerase II transcriptional factors are modified by both phosphorylation and Glc-NAcylation. We have recently shown that enhancer of split (E1A-binding protein) 4 (E4) is phosphorylated and GlcNAcylated on Ser 86 by casein kinase II and OGT, respectively (9). Here we report that C/EBPβ is also phosphorylated on Thr188, Ser184, and Thr179 by MAPK and GSK3β. Phosphorylation of these sites is known to activate C/EBPβ transcriptional activity (15, 25). GlcNAcylation of C/EBPβ on Ser 180 and Ser 181 may affect the transcriptional activity of this factor. C/EBPβ is a transcription factor that participates in the adipocyte differentiation program (22). Phospho-C/EBPβ is known to play a role in the modulation of adipocyte differentiation (22). Increasing evidence suggests that modification of C/EBPβ by phosphorylation and GlcNAcylation may have a role in the modulation of adipocyte differentiation.

We have also shown that increasing the GlcNAcylation of C/EBPβ decreases its DNA binding and transactivation activities and subsequently delays the adipocyte differentiation program. C/EBPβ is a transcription factor that participates in the adipocyte differentiation program (22). Phospho-C/EBPβ is known to play a role in the modulation of adipocyte differentiation (22). Increasing evidence suggests that modification of C/EBPβ by phosphorylation and GlcNAcylation may have a role in the modulation of adipocyte differentiation.

We have recently shown that enhancer of split (E1A-binding protein) 4 (E4) is phosphorylated and GlcNAcylated on Ser 86 by casein kinase II and OGT, respectively (9). Here we report that C/EBPβ is also phosphorylated on Thr188, Ser184, and Thr179 by MAPK and GSK3β. Phosphorylation of these sites is known to activate C/EBPβ transcriptional activity (15, 25). GlcNAcylation of C/EBPβ on Ser 180 and Ser 181 may affect the transcriptional activity of this factor. C/EBPβ is a transcription factor that participates in the adipocyte differentiation program (22). Phospho-C/EBPβ is known to play a role in the modulation of adipocyte differentiation (22). Increasing evidence suggests that modification of C/EBPβ by phosphorylation and GlcNAcylation may have a role in the modulation of adipocyte differentiation.
GlcNAcylation of C/EBPβ

GlcNAcylation, whereas phosphorylation and GlcNAcylation often reciprocally regulate the activities of these transcriptional factors (32). c-Myc is a helix-loop-helix leucine zipper transcription factor. Ser62 and Thr58 are identified as phosphorylation sites, and phosphorylation on Ser62 by MAPK primes the phosphorylation on Thr58 by GSK3β. Thr246 is also identified as the major GlcNAcylation site of c-Myc. Thr58 is located in the transactivation domain, and the competitive occupancy at this amino acid (Thr58) plays an important role in regulation of the functions of c-Myc (33). FoxO1 is an important transcription factor regulating broad gene expression programs including metabolism, cell cycle, longevity, and stress response. The phosphorylation of FoxO1 by insulin signaling pathway has been well characterized, and phosphorylations on Thr246/Thr256/Thr261 exclude the FoxO1 from nucleus. FoxO1 is also GlcNAcylated, and the GlcNAcylation sites are mapped to Thr317/Ser350/Thr348/Ser656. Further evidence demonstrates that the phosphorylation sites do not overlap directly with but are adjacent to the GlcNAcylation sites, suggesting a complex interplay between phosphorylation and GlcNAcylation of FoxO1. Interestingly, the GlcNAcylation of FoxO1 increases its transactivation capability in response to high glucose (24).

Our previous data demonstrate that the transcription factor C/EBPβ is sequentially phosphorylated on Thr188/Ser184/Thr179, which is similar to c-Myc; phosphorylation on Thr188 by MAPK or CyclinA/cdk2 primes the phosphorylations on Ser184/Thr179 by GSK3β; and phosphorylations on Thr188/Ser184/Thr179 are required for the acquisition of DNA binding activity by C/EBPβ. In this paper we demonstrate that C/EBPβ is also modified by O-GlcNAc (Fig. 1), and the GlcNAcylation sites are on Ser180 and Ser181 (Fig. 2), which are very close to its phosphorylation sites (Thr188, Ser184, and Thr179) and both of which are located in the RD of C/EBPβ. Although GlcNAcylation itself does not affect the DNA binding activity in vitro (Fig. 3), it prevents the phosphorylations (Fig. 4). C/EBPβ is GlcNAcylated during 3T3-L1 adipocyte differentiation (Fig. 5), and elevation of GlcNAcylation significantly decreases the phosphorylation (Fig. 6) as well as the DNA binding activity of C/EBPβ in vivo (Fig. 7A), subsequently delaying the adipocyte differentiation program (Fig. 7, B and C). Importantly mutations on Ser180 and Ser181 significantly increase the transactivation activity of C/EBPβ detected by C/EBPα promoter reporter assay (Fig. 7B), indicating that blocking of GlcNAcylation favors the phosphorylations. We conclude that GlcNAcylation and phosphorylation act in a competitive manner and by alternative occupancy at adjacent sites to regulate the function of C/EBPβ, which is similar to the situation with FoxO1.

REFERENCES

1. Cao, Z., Umek, R. M., and McKnight, S. L. (1991) Genes Dev. 5, 1538–1552
2. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E., and Schibler, U. (1990) Genes Dev. 4, 1541–1551
3. Oh, H. S., and Smart, R. C. (1998) J. Invest. Dermatol. 110, 939–945
4. Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. (1998) Genes Dev. 12, 1907–1916
5. Katz, S., Kowenz-Leutz, E., Müller, C., Meese, K., Ness, S. A., and Leutz, A. (1993) EMBO J. 12, 1321–1332
6. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2593–2597
7. Liu, S., Crouzier, C., Arizmendi, C., Harada-Shiba, M., Ren, J., Poli, V., Hanson, R. W., and Friedman, J. E. (1999) J. Clin. Invest. 103, 207–213
8. Greenbaum, L. E., Li, W., Cressman, D. E., Peng, Y., Ciliberto, G., Poli, V., and Taub, R. (1998) J. Clin. Invest. 102, 996–1007
9. Seagroves, T. N., Kranic, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J., and Rosen, J. M. (1998) Genes Dev. 12, 1917–1928
10. Scott, L. M., Civen, C. I., Rorth, P., and Friedman, A. D. (1992) Blood 80, 1725–1735
11. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scapa, S., Bellavia, D., and Lattanzio, G. (1995) EMBO J. 14, 1932–1941
12. Wü, Z., Xie, Y., Bucher, N. L., and Farmer, S. R. (1995) Genes Dev. 9, 2350–2363
13. Yeh, W. C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Genes Dev. 9, 168–181
14. Tang, Q. Q., Gronberg, M., Huang, H., Kim, J. W., Otto, T. C., Pandey, A., and Lane, M. D. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 9766–9771
15. Li, X., Kim, J. W., Gronberg, M., Urlaub, H., Lane, M. D., and Tang, Q. Q. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 11597–11602
16. Kim, J. W., Tang, Q. Q., Li, X., and Lane, M. D. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 1800–1804
17. Torres, C. R., and Hart, G. W. (1984) J. Biol. Chem. 259, 3308–3317
18. Holt, G. D., and Hart, G. W. (1986) J. Biol. Chem. 261, 8049–8057
19. Hart, G. W., Housley, M. P., and Lawson, C. (2007) Nature 446, 1017–1022
20. Kreppel, L. K. Blomberg, M. A., and Hart, G. W. (1997) J. Biol. Chem. 272, 9308–9315
21. Gao, Y., Wells, L., Comer, F. I., Parker, G. J., and Hart, G. W. (2001) J. Biol. Chem. 276, 9838–9845
22. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) J. Biol. Chem. 268, 10416–10424
23. Yang, X., Zhang, F., and Kudlow, J. E. (2002) Cell 110, 69–80
24. Houseley, M. P., Rodgers, J. T., Udeshi, N. D., Kelly, T. J., Shabanowitz, J., Hunt, D. F., Puigserver, P., and Hart, G. W. (2008) J. Biol. Chem. 283, 16283–16292
25. Ray, A., and Ray, B. K. (1994) Mol. Cell. Biol. 14, 4324–4332
26. Cesena, T. I., Cui, T. X., Subramanian, L., Fulton, C. T., Iguchi-Lluhi, J. A., Kwok, R. W., and Scharff, I. (2008) Mol. Cell. Endocrinol. 289, 94–101
27. Eaton, E. M., and Sealy, L. (2003) J. Biol. Chem. 278, 33416–33421
28. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
29. Khidekel, N., Arndt, S., Lamerre-Vincent, N., Lippert, A., Poulin-Kerstien, K., Ramakrishnan, B., Qasba, P. K., and Hsieh-Wilson, L. C. (2003) I. Am. Chem. Soc. 125, 16162–16163
30. Student, A. K., Hsu, R. Y., and Lane, M. D. (1980) J. Biol. Chem. 255, 4745–4750
31. Wang, Z., Park, K., Comer, F. I., and Hsieh-Wilson, L. C., Saudek, C. D., and Hart, G. W. (2009) Diabetes 58, 309–317
32. Comer, F. I., and Hart, G. W. (2001) Biochemistry 40, 7845–7852
33. Kamemura, K., Hayes, B. K., Comer, F. I., and Hart, G. W. (2002) J. Biol. Chem. 277, 19229–19235