Sequential phosphorylation of CCAAT enhancer-binding protein β by MAPK and glycogen synthase kinase 3β is required for adipogenesis

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CCAAT enhancer-binding protein (C/EBP)β, C/EBPα, and peroxisome proliferator activated receptor (PPAR)γ act in a cascade where C/EBPβ activates expression of C/EBPα and PPARγ, which then function as pleiotropic activators of genes that produce the adipocyte phenotype. When growth-arrested 3T3-L1 preadipocytes are induced to differentiate, C/EBPβ is rapidly expressed but still lacks DNA-binding activity. After a long (14-hour) lag, glycogen synthase kinase 3β enters the nucleus, which correlates with hyperphosphorylation of C/EBPβ and acquisition of DNA-binding activity. Concurrently, 3T3-L1 preadipocytes synchronously enter S phase and undergo mitotic clonal expansion, a prerequisite for terminal differentiation. *Ex vivo* and *in vitro* experiments with C/EBPβ show that phosphorylation of Thr-188 by mitogen-activating protein kinase "primes" C/EBPβ for subsequent phosphorylation on Ser-184 and Thr-179 by glycogen synthase kinase 3β, acquisition of DNA-binding function, and transactivation of the C/EBPα and PPARγ genes. The delayed transactivation of the C/EBPα and PPARγ genes by C/EBPβ appears necessary to allow mitotic clonal expansion, which would otherwise be prevented, because C/EBPα and PPARγ are antimitotic.

3T3-L1 preadipocyte | cell cycle | differentiation | mitotic clonal expansion

CCAAT enhancer-binding protein β (C/EBPβ) is expressed early in the adipocyte differentiation program, first initiating mitotic clonal expansion (MCE) (1, 2) and later activating expression of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) (3–6), pleiotropic activators of adipocyte genes (3, 4, 7, 8). Both MCE and expression of C/EBPα and PPARγ are required for differentiation (1, 2, 9). When treated with differentiation inducers, growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle, undergo approximately two rounds of mitosis, then exit the cell cycle and enter the terminal stages of differentiation. Transcriptional activation of the C/EBPα and PPARγ genes is induced by the interaction of C/EBPβ with C/EBP regulatory elements in these gene promoters (3–6).

Although expression of C/EBPβ occurs within 2 h of induction of differentiation, acquisition of DNA-binding activity and thus transcription of the C/EBPα and PPARγ genes are delayed (10). Acquisition of DNA-binding activity begins after a long lag ($\approx$14 h), concurrent with the entry of S phase at the onset of MCE and transcription of the C/EBPα and PPARγ genes (ref. 10 and Fig. 1). This lag appears necessary, because C/EBPα and PPARγ are antimitotic (11–15), and their premature expression would otherwise prevent the MCE required for differentiation. To elucidate the mechanism by which C/EBPβ acquires DNA-binding activity, we considered the possibility that covalent modification of C/EBPβ occurs during this time window.

Several lines of evidence indicated that C/EBPβ can be phosphorylated *in vitro* by a variety of kinases, including PKA (16), PKC (16), mitogen-activated protein kinase (MAPK) (17), and Ca^{2+}-calmodulin-dependent kinase II (18). However, functional effects were not observed. We found (10) that treatment of nuclear extracts from 3T3-L1 preadipocytes with alkaline phosphatase disrupted the DNA-binding activity of C/EBPβ. Thr-188 in C/EBPβ was implicated as a phosphorylation site of MAPK in the oncogenic ras signaling pathway (17, 19) and was also found to play roles in keratinocyte survival and skin tumorigenesis (19) and in C/EBPβ-dependent gene expression in response to IFN-γ (20). Phosphorylation of C/EBPβ on Ser-105 in rat C/EBPβ (Thr-217 in mouse C/EBPβ) by ribosomal S kinase appears to be required for hepatocyte proliferation during liver regeneration and for the proliferative response of hepatocytes to TGFα (21).

The present paper shows, both *ex vivo* and *in vitro*, that C/EBPβ is sequentially phosphorylated by MAPK (on Thr-188) and by glycogen synthase kinase 3β (GSK3β) (on Ser-184 and Thr-179) early in the adipocyte differentiation program. Moreover, phosphorylation by MAPK and GSK3β *in vitro* leads to the acquisition of DNA-binding function.

Materials and Methods

Cell Culture, Induction of Differentiation, and Transfection of 3T3-L1 Preadipocytes. Differentiation of postconfluent 3T3-L1 preadipocytes (designated day 0) was as described (22). The MAPK (U0126, Calbiochem) and GSK3β (SB216763, Calbiochem) inhibitors (20 μM) were added 1 h before and at the time of induction of differentiation. U0126 was added again 24 h later. Cell number was determined on day 4 and Oil-red-O staining (2) on day 8. Transfections were performed with proliferating preconfluent (at 40–50% confluent cell density) 3T3-L1 preadipocytes by the calcium phosphate coprecipitation method (23).

EMSA and Chromatin Immunoprecipitation (ChIP) Analysis. Nuclei were isolated and nuclear extracts prepared by using 1× NUN buffer (24) containing 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM Hepes (pH 7.9), and 1 mM DTT. EMSA was performed essentially as described (10). For supershift experiments, 1 μl of antiserum ($\approx$5 μg of IgG protein) was added to the reaction mixture before addition of the labeled probe. The labeled probe included a double-stranded oligonucleotide corresponding to the sequence of the C/EBP regulatory element in the C/EBPα gene promoter (4), G_{191}CGTTGCGCCACGATCTCTC$_{172}$. ChIP analysis was performed essentially as described (25). 3T3-L1 preadipocytes were induced to differentiate with or without MAPK (U0126) and GSK3β (SB216763) inhibitors; 24 h later, ChIP analysis was performed with primers flanking C/EBP-binding site in the 422/aP2 promoter: (*i*) CCTCCACA-

Abbreviations: C/EBP, CCAAT enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; MCE, mitotic clonal expansion; MAP kinase, mitogen-activated protein kinase; LC, liquid chromatography; MS/MS, tandem MS; LAP, liver activator protein; GSK3β, glycogen synthase kinase 3β; ChIP, chromatin immunoprecipitation.

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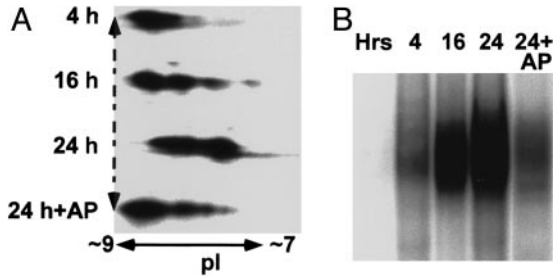

Fig. 1. Changes in phosphorylation state and DNA-binding activity of C/EBPβ during differentiation. Two-day postconfluent 3T3-L1 preadipocytes were induced to differentiate nuclear extracts prepared at 4, 16, and 24 h after induction (treated or not with alkaline phosphatase) and then subjected to (*A*) 2D gel analysis and immunoblotting with anti-C-terminal C/EBPβ antibody and (*B*) EMSA to assess DNA-binding activity using a oligonucleotide probe corresponding to the C/EBP regulatory element in the proximal promoter of the C/EBPα gene.

ATGAGGCAAATC and (*ii*) CTGAAGTCCAGATAGCTC. PCR products were analyzed with 2% agarose.

Immunoblotting. At the times indicated, cell lysates were prepared as described (10), subjected to SDS/PAGE, and immunoblotted with the appropriate antibodies. C/EBPβ, C/EBPα, and 422/aP2 antibodies were prepared in this laboratory, and the antibody against PPARγ was from Mitchell Lazar (University of Pennsylvania, Philadelphia). Antibody against *p*-Thr-188-C/EBPβ was from Cell Signaling Technology (Beverley, MA). Antibodies against MAPK and phospho-MAPK (Thr-202/Tyr-204) were from Upstate Biotechnology (Lake Placid, NY) and GSK3β antibody from Santa Cruz Biotechnology.

2D Isoelectric Focusing/SDS/PAGE. Growth-arrested 3T3-L1 preadipocytes were induced to differentiate as above and nuclei prepared (10) at 4, 16, and 24 h and lysed in buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 40 mM Tris base. After mixing with rehydration buffer containing 8 M urea, 2% CHAPS, and 2% immobilized pH gradient buffer (pH 3–10), the sample was soaked in IPG strips (pH 3–10), isoelectrofocused by using an IPGphor isoelectric focusing system (Amersham Pharmacia Biotech). Proteins were then further resolved by 5–12% SDS/PAGE and C/EBPβ detected by immunoblotting. Alkaline phosphatase treatment was performed as described (4).

Purification, In-Gel Digestion, and Nano-Liquid Chromatography (LC)–Tandem MS (MS/MS) Analysis of Endogenous C/EBPβ. A C/EBPβ immunoaffinity column was prepared by coupling the purified C-terminal C/EBPβ antibody (against CTLRNLFKQLPEPLLASAGH-OH) to a protein A column using an ImmunePure rProtein A IgG Plus Orientation Kit (Pierce Biotechnology). One hundred 15-cm dishes of 3T3-L1 preadipocytes were induced to differentiate, after which nuclear extracts were prepared 4 and 24 h later by the NUN method (above). C/EBPβ protein was purified by using the immunoaffinity column according to the manufacturer's instructions and then subjected to SDS/PAGE. The band containing C/EBPβ (38 kDa) was excised from the gel, digested with trypsin, and analyzed by automated nanoLC-MS/MS, as described (26).

***In Vitro* Phosphorylation and MS Analysis of Synthetic Peptides.** Two micrograms of each peptide (synthesized by Biopeptide, San Diego) were incubated either: (*i*) with activated MAPK (Calbiochem) in buffer containing 100 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 20 μCi [γ^{32}P] ATP (1 Ci = 37 GBq), or (*ii*) with GSK3β (Calbiochem) in buffer containing 20 mM Tris·HCl, pH 7.5, 10 mM MgCl2, 5 mM DTT, 200 μM ATP (20 μCi [γ^{32}P] ATP) at 30°C for 30 min. The peptide was purified by using a C_{18} SepPak column (27), and incorporation of [^{32}P] into the phosphopeptide was determined. Identical reactions, but with unlabeled ATP, were processed as above. After elution, the peptides were dried down and redissolved in 50 μl of acetonitrile and 5% formic acid 1:1 (vol/vol). Approximately 1 μl of the redissolved peptide was analyzed by nanoelectrospray ionization MS. All spectra were obtained in positive ion mode. A potential of 0.7–1.0 kV was applied to the nanospray needle obtained from Proxeon Biosystems (Odense, Denmark).

***In Vitro* Phosphorylation and Analysis of Full-Length C/EBPβ.** The AAA mutant (Thr-179, Ser-184, and Thr-188→Ala) was constructed by using the QuickChange site-directed mutagenesis kit (Invitrogen). The WT or AAA mutant C/EBPβ(LAP) (LAP, liver activator protein) was cloned into pGEX-4T (Amersham Pharmacia Biotech), transformed into *Escherichia coli* [strain BL21(DE3)pLysS; Novagen] and GST-C/EBPβ prepared. Two micrograms of WT or AAA mutant C/EBPβ was incubated with activated MAPK and/or GSK3β in 100 mM Tris·HCl (pH 7.5)/10 mM $MgCl_2$/1 mM EGTA/5 mM DTT/20 μCi [γ^{32}P] ATP at 30°C for 30 min. ^{32}P-C/EBPβ was detected by autoradiography after SDS/PAGE. To assess DNA-binding activity (EMSA), an identical reaction mixture with unlabeled ATP was used. To identify amino acids phosphorylated by MAPK and/or GSK3β, C/EBPβ was purified by SDS/PAGE and the C/EBPβ band cut out and subjected to in-gel digestion and MS analysis.

Results

C/EBPβ Undergoes Phosphorylation Correlated with Acquisition of DNA-Binding Activity During Differentiation. Experiments were conducted to verify and extend our previous studies (10), suggesting that C/EBPβ undergoes changes in phosphorylation state early in the adipocyte differentiation program. Nuclear extracts prepared from preadipocytes at 4, 16, and 24 h after induction of differentiation were subjected to isoelectric focusing/SDS/PAGE (2D gel electrophoresis) and then immunoblotted with anti-C/EBPβ antibody. Four hours after induction, C/EBPβ/LAP (Fig. 1*A*) and C/EBPβ/LIP (not shown) exhibited high isoelectric points (pI ≈9). By 16 h after induction, however, a large fraction (≈50%) of C/EBPβ/LAP converted to forms with more acidic pIs and by 24 h, virtually all C/EBPβ/LAP shifted to the lower pI forms. These changes were correlated with the acquisition of DNA-binding activity as measured by EMSA with an oligonucleotide probe corresponding to the C/EBP regulatory element in the C/EBPα gene (Fig. 1*B* and ref. 10). Four hours after induction, C/EBPβ possessed little DNA-binding activity; however, by 16–24 h, DNA-binding activity was acquired (Fig. 1*B*). Previously, we showed that acquisition of DNA binding activity begins ≈14 h after induction as the preadipocytes synchronously traverse the G_1-S checkpoint (10).

To verify that the shifts to more acidic pIs at 16 and 24 h were due to phosphorylation of C/EBPβ, nuclear extracts were treated with alkaline phosphatase before 2D gel electrophoresis. Phosphatase treatment caused reversal to forms with less acidic pIs approaching those at the 4-h time point (Fig. 1*A*). The shifts at 16 and 24 h to forms with multiple pIs suggested multiple phosphorylation sites. That phosphorylation of C/EBPβ is responsible for the acquisition of DNA-binding activity was verified by EMSA. As illustrated in Fig. 1*B* and previously inferred (10) phosphatase treatment of nuclear extract prepared 24 h after induction of differentiation disrupted DNA-binding activity. Thus, acquisition of DNA-binding activity appears to depend upon phosphorylation of C/EBPβ at ≈14 h, as the cells enter S phase, and reaches a maximum at 24 h (10).

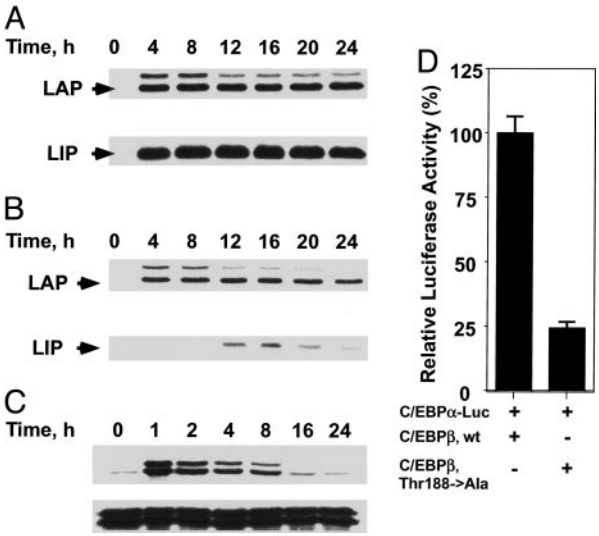

Fig. 2. Expression and phosphorylation of C/EBPβ and MAPK during differentiation. (*A–C*) Two-day postconfluent 3T3-L1 preadipocytes were induced to differentiate. At the times indicated, cell extracts were prepared and immunoblotted with antibodies directed against (*A*) the C-terminal peptide of C/EBPβ, (*B*) phospho-Thr-188 C/EBPβ, and (*C*) phospho-MAPK (*Upper*) and MAPK (*Lower*). (*D*) Transactivation of a C/EBPα promoter-luciferase reporter gene by WT or mutant (Thr-188→Ala) C/EBPβ expression vector.

Phosphorylation of the Thr-188 MAPK Site in C/EBPβ During Differentiation. To determine whether phosphorylation of C/EBPβ at this Thr-188 correlates with acquisition of DNA-binding activity, the kinetics of phosphorylation after induction was monitored by with antiphosphopeptide antibody to the MAPK site at phospho-Thr-188. Parallel gels were immunoblotted with antibody to a peptide corresponding to the C terminus of C/EBPβ (Fig. 2*A*). Consistent with previous findings (10), C/EBPβ was rapidly ($\leq$4 h) expressed after induction to level that remained constant for at least 24 h. Phosphorylation of Thr-188 in C/EBPβ LAP occurred immediately ($\leq$4 h) after induction (Fig. 2*B*).

That MAPK is responsible for the rapid phosphorylation of C/EBPβ LAP after induction is supported by the fact that MAPK itself is rapidly phosphorylated (Fig. 2*C*) and thereby activated after induction remaining relatively constant for the next 8 h (Fig. 2*C*). Despite phosphorylation at Thr-188, however, DNA-binding activity was not acquired at 4 h but was delayed until 14–16 h after induction (10) (Fig. 1*B*). Thus, phosphorylation at Thr-188 alone is insufficient to affect DNA-binding activity.

Although phosphorylation of Thr-188 alone is insufficient for acquisition of DNA-binding activity, that this site is required is suggested by the fact that mutation of Thr-188 disrupts transactivation by C/EBPβ. Thus, mutation of Thr-188 disrupted reporter gene expression driven by C/EBPβ (Fig. 2*D*).

Identification of Two Additional Sites in C/EBPβ That Undergo Phosphorylation Later in the Differentiation Program. Because 2D gel analysis (Fig. 1*A*) indicated that C/EBPβ is phosphorylated at multiple sites during MCE, investigations were conducted to identify the phosphorylation sites. 3T3-L1 preadipocytes were induced to differentiate and nuclear extracts prepared at 4 and 24 h after induction. C/EBPβ was purified from the nuclear extracts by using an immunoaffinity matrix, elution being accomplished with low pH buffer. Purified C/EBPβ was separated by SDS/PAGE, and the band corresponding to C/EBPβ(LAP) was excised and digested with trypsin. Automated nanoLC-MS/MS analysis of the digested gel band identified eight peptides that correspond to a total sequence coverage of 40%. One triply charged peptide (m/z 1,094.48) showed a characteristic loss of phosphoric acid (loss of 98 Da) and was identified as a singly phosphorylated peptide (data not shown). The MS/MS data obtained from automated nanoLC-MS/MS analysis did not provide sufficient sequence information to localize the exact phosphorylation site in C/EBPβ. However, manual selection of the identified phosphopeptide (m/z 1,094.48) during an LC-MS/MS run, combined with manual peak parking, showed that this peak corresponded to a mixture of two singly phosphorylated peptides that were phosphorylated on Thr-179 (Fig. 3*A*) or Ser-184 (Fig. 3*B*). The presence of dehydroaminobutyric acid and dehydroalanine, respectively, was the result of a gas phase β-elimination reaction. Both of these are phosphorylation sites not previously identified in C/EBPβ. These residues (Thr-179 and Ser-184) are located in a region rich in Pro and Gly residues just N-terminal to the Thr-188 MAPK phosphorylation site and thus have the characteristic of sites phosphorylated by GSK3β (Fig. 3*C*).

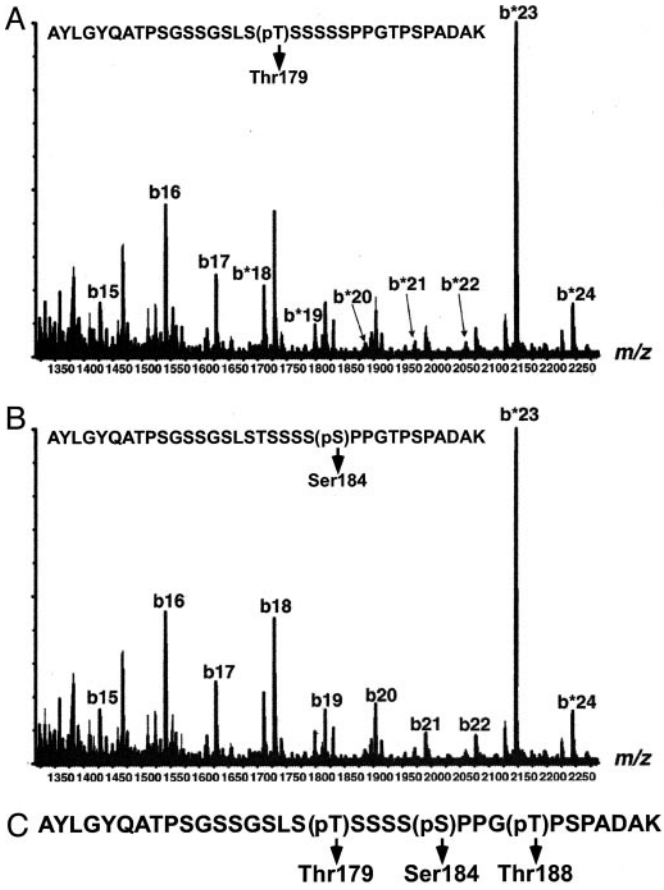

Fig. 3. Identification of phosphorylation sites in C/EBPβ during differentiation. (*A* and *B*) Nuclear extracts were prepared before and 24 h after induction of differentiation. C/EBPβ was isolated and separated by SDS/PAGE. The band containing C/EBPβ (38 kDa) was excised from the gel, digested by trypsin, and analyzed by automated nanoLC-MS/MS as described (26). Ions labeled with an asterisk were generated from the peptide in which the phosphoserine and phosphothreonine were converted into dehydroalanine and dehydroaminobutyric acid by β-elimination, respectively. (*A*) Partial MS/MS spectrum of one of the species in which the phosphorylation site was localized to Thr-179. (*B*) Partial MS/MS spectrum of the second species where the phosphorylation site was localized to Ser-184. (*C*) Amino acid sequence of the tryptic peptide identifying the phosphorylation sites in Fig. 2*B* (Thr-188) and in *A* (Thr-179) and *B* (Ser-184) above.

Phosphorylation of WT and Mutant Peptides by MAPK and GSK3β. The evidence presented above suggested that phosphorylation of Thr-188 by MAPK may be the first event in a phosphorylation cascade. To test the possibility that phosphorylation of Thr-188

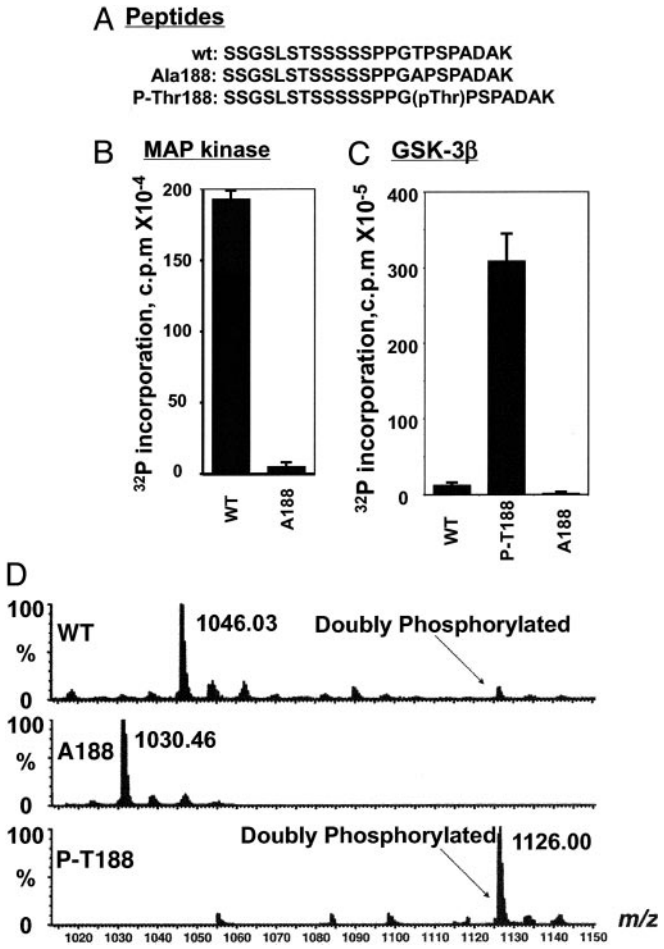

Fig. 4. Phosphorylation of WT and mutant peptides by MAPK and GSK3β. (*A*) Amino acid sequences of the WT and mutant (Thr-188→Ala and Thr-188→phospho-Thr-188) synthetic peptides used as substrates for MAPK (*B*) and GSK3β (*C*) below. Peptides were incubated individually with MAPK or GSK3β and [γ-^{32}P]ATP/Mg, after which ^{32}P-incorporation into the peptide was determined. (*D*) MS analysis of WT and mutant peptides phosphorylated *in vitro* by GSK3β. WT, WT peptide; Ala-188, Thr-188→Ala peptide; and P-Thr-188, phospho-Thr-188 peptide.

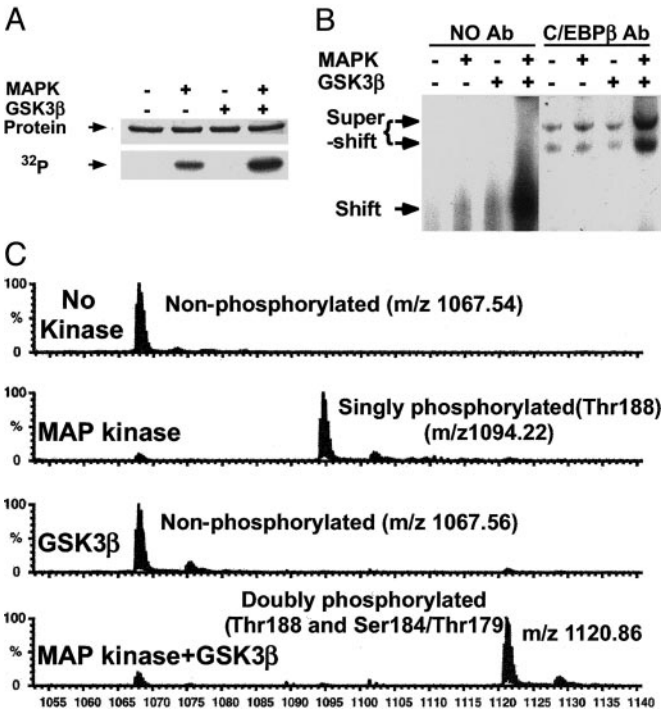

Fig. 5. *In vitro* phosphorylation of full-length C/EBPβ by MAPK and GSK3β leads to acquisition of DNA-binding activity. (*A*) [^{32}P] labeling of WT C/EBPβ, (*B*) DNA-binding activity assessed by EMSA, and (*C*) MS analysis of C/EBPβ after *in vitro* phosphorylation by MAPK and/or GSK3β. After incubation with MAPK and/or GSK3β and ATP, recombinant full-length GST-C/EBPβ(LAP) was digested by trypsin and analyzed by MS. The masses (m/z) of tryptic peptide products are shown.

by MAPK is required for subsequent hyperphosphorylation at Thr-179 and Ser-184, three peptides (WT, a Thr-188→Ala mutant peptide, and a P-Thr-188 phosphopeptide; Fig. 4*A*) were synthesized and tested *in vitro* as substrates for MAPK and GSK3β by using [γ-^{32}P] ATP as phosphoryl donor. Mutation of Thr-188 to Ala almost completely abolished phosphorylation by MAPK (Fig. 4*B*). This is consistent with the fact that (*i*) the same mutation (Thr-188→Ala) disrupts transactivation of the C/EBPα promoter by C/EBPβ (Fig. 2*D*) and (*ii*) the rapid phosphorylation of C/EBPβ (4 h after induction; Fig. 2*B*) closely follows phosphorylation/activation of MAPK (Fig. 2*C*).

The two phosphorylation sites in C/EBPβ identified above, i.e., Thr-179 and Ser-184, are potential GSK3β phosphorylation sites. GSK3β possesses unique substrate specificity, in that its substrates must initially undergo phosphorylation by another protein kinase at a serine or threonine located approximately four residues C-terminal to the GSK3β phosphorylation site (28). The two phosphorylation sites, i.e., Thr-179 and Ser-184 and the MAPK site at Thr-188 in C/EBPβ, fit this consensus requirement (Fig. 3*C*). To determine whether Thr-179 and Ser-184 can be phosphorylated by GSK3β and whether phosphorylation of Thr-188 (Fig. 2*B*) by MAPK is required, the three peptides (Fig. 4*A*) were tested *in vitro* as substrates for GSK3β by using [γ-^{32}P] ATP as phosphoryl donor.

Consistent with the predicted phosphorylation pattern for GSK3β substrates, phospho-Thr-188 peptide was an excellent substrate, whereas the WT and Thr-188→Ala mutant peptides were poor substrates (Fig. 4*C*). Although far less active than the phospho-Thr-188 peptide, the WT peptide was 10 times more active as a substrate than the Thr-188→Ala mutant peptide. To identify the amino acid(s) phosphorylated by GSK3β, the same reaction was performed with unlabeled ATP, after which the peptides were purified and analyzed by MS. MS analysis of the peptides after phosphorylation by GSK-3β showed that almost all of the phospho-Thr-188 peptide became doubly phosphorylated (Fig. 4*D*). However, only a small fraction of the WT peptide became doubly phosphorylated, most remaining in the unphosphorylated form (Fig. 4*D*). No phosphorylation of the mutant Thr-188→Ala peptide was detected (Fig. 4*D*). Sequence analysis of the product of phospho-Thr-188 peptide showed that the product contained two populations, one phosphorylated on Thr-188 and Thr-179 (Fig. 8*A*, which is published as supporting information on the PNAS web site) and another phosphorylated on Thr-188 and Ser-184 (Fig. 8*B*). Together, these results show that phosphorylation of Thr-188 (by MAPK) serves to prime phosphorylation at Thr-179 and Ser-184 by GSK3β.

Translocation of GSK3β from Cytoplasm to Nucleus Concomitant with Hyperphosphorylation and Acquisition of DNA-Binding Activity. Previous studies showed that C/EBPβ translocates to the nucleus immediately after induction of differentiation (10). Because there is a 12- to 14-h lag before C/EBPβ acquires DNA-binding activity (ref. 10 and Fig. 1*B*) and undergoes hyperphosphorylation at Thr-179 or Ser-184, it was of interest to determine

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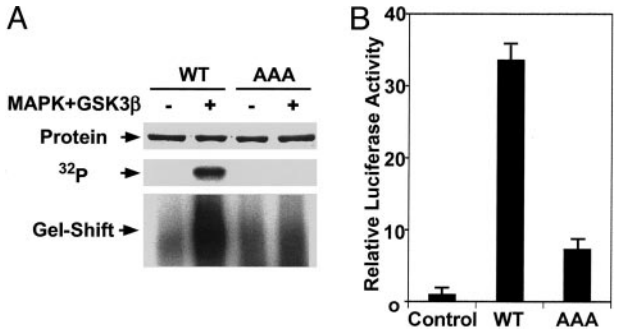

Fig. 6. Mutation of Thr-188, Ser-184, and Thr-179 to Ala abolishes DNA-binding and transactivation activities of C/EBPβ. (*A*) recombinant WT or AAA mutant C/EBPβ was incubated with MAPK and GSK3β after which [^{32}P] labeling and DNA-binding activity (by EMSA) of C/EBPβ were assessed. (*B*) Transactivation of a C/EBPα promoter-luciferase reporter gene by WT or AAA mutant (Thr-188, Ser-184, Thr-179→Ala) C/EBPβ expression vector.

whether the putative kinase, GSK3β, is present in the nucleus at this point in the program. Intracellular localization experiments (Fig. 9, which is published as supporting information on the PNAS web site) revealed that before 12 h after induction, GSK3β does not exhibit nuclear localization. However, between 12 and 16 h, ≈25% of GSK3β enters the nucleus, after which the level remains constant. Thus, the kinetics of translocation of GSK3β into the nucleus is consistent with its suspected role in the hyperphosphorylation of C/EBPβ (Fig. 1*A*) and the acquisition of DNA-binding activity (Fig. 1*B* and ref. 10).

***In Vitro* Phosphorylation of Full-Length Recombinant C/EBPβ on Thr-188 and Ser-184/Thr-179 Gives Rise to DNA-Binding Activity.** Full-length C/EBPβ(LAP) was overexpressed in *E. coli* as a GST fusion protein, purified, and incubated either without or with MAPK, GSK3β, or both in the presence of [γ-^{32}P] ATP. C/EBPβ was phosphorylated by MAPK alone but not by GSK3β alone (Fig. 5*A*). Incubation with MAPK, then GSK3β, however, increased the extent of phosphorylation by ≈2-fold relative to that by MAPK alone (Fig. 5*A*). This is consistent with *in vitro* phosphorylation of synthetic peptide substrates corresponding to the phosphorylated region in C/EBPβ (Fig. 4).

To identify the amino acids in full-length C/EBPβ(LAP) that were phosphorylated *in vitro*, experiments similar to those above were performed. After separation by SDS/PAGE, the band corresponding to C/EBPβ(LAP) was excised, digested with trypsin, and subjected to MS analysis. Only one triply charged singly phosphorylated species was found with MAPK alone (Fig. 5*C*); this phosphorylation site was identified as Thr-188 (Fig. 10, which is published as supporting information on the PNAS web site). Analysis of the peptide phosphorylated by MAPK and GSK3β together revealed a doubly phosphorylated species (Fig. 5*C*). MS/MS showed two different populations of doubly phosphorylated peptides, one phosphorylated on Thr-188 and Ser-184 (Fig. 11, which is published as supporting information on the PNAS web site) and another phosphorylated on Thr-188 and Ser-179 (results similar to those shown in Fig. 11; not shown). No phosphorylation product was detected with GSK3β alone (Fig. 5*C*). These results show that full-length C/EBPβ is phosphorylated on Thr-188 by MAPK, and that this phosphorylation serves as the priming site for phosphorylation of Ser-184/Thr-179 by GSK3β.

An identical experiment was performed, after which DNA-binding activity was assessed by gel-shift analysis with a labeled oligonucleotide corresponding to the C/EBP-binding site in the C/EBPα gene promoter. DNA-binding activity was acquired only after C/EBPβ(LAP) was phosphorylated by both MAPK and GSK3β (Fig. 5*B*). Unphosphorylated C/EBPβ(LAP) or C/EBPβ(LAP) phosphorylated by MAPK did not exhibit significant DNA-binding activity. Consistent with these results, mutation of the three phosphorylation sites (Thr-188→Ala, Ser-184→Ala and Thr-179→Ala; AAA mutant) prevented phosphorylation by MAPK and GSK3β, the acquisition of DNA-binding activity (Fig. 6*A*), and transactivation function (Fig. 6*B*). Together, these results show that phosphorylation of C/EBPβ(LAP) on Thr-188 is required for further phosphorylation on Ser-184/Thr-179 by GSK3β and for acquisition of DNA-binding activity.

Effect of MAPK and GSK3β Inhibitors on DNA Binding by C/EBPβ and Adipogenesis. To determine whether inhibition of the MAPK or GSK3β affects the cellular functions of C/EBPβ, 3T3-L1 preadipocytes were treated with U0126 (a MEK/MAPK inhibitor) or SB216763 (a GSK3β inhibitor) at the time of induction of differentiation. At various times after induction of differentiation the DNA-binding activity of C/EBPβ, MCE, expression of

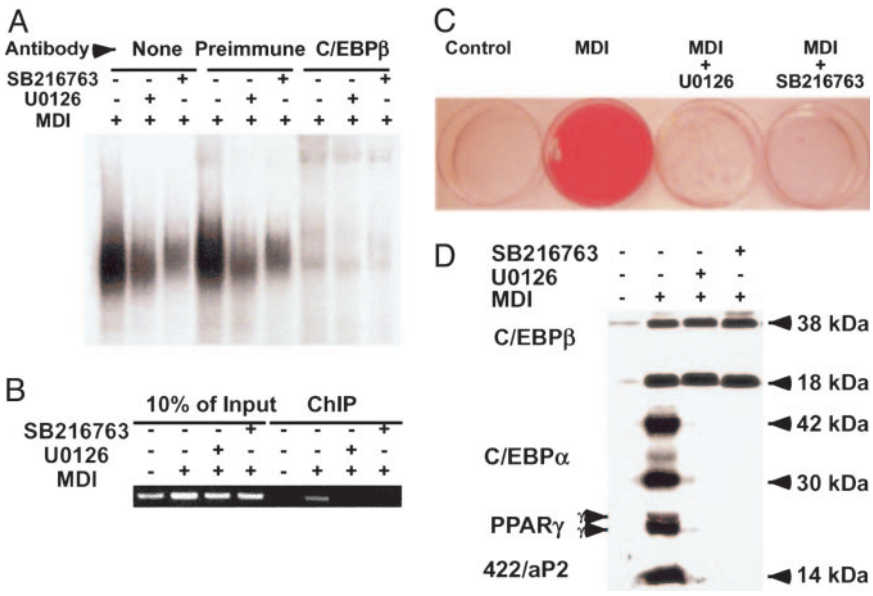

Fig. 7. Inhibition of MAPK or GSK3β disrupts DNA-binding activity of C/EBPβ and differentiation. U0126 (20 μM, MAPK inhibitor) or SB216763 (20 μM, GSK3β inhibitor) was added 60 min before and at the time of induction. (*A*) Twenty-four hours after induction, DNA-binding activity of C/EBPβ was determined by EMSA, and (*B*) ChIP analysis was performed with oligonucleotide primers bracketing the C/EBP-binding site in the 422/aP2 gene promoter. (*C*) On day 8, cells were stained with Oil-red O, and (*D*) expression of C/EBPβ was assessed by Western blotting on day 1 and adipocyte markers (C/EBPα, PPARγ, and 422/aP2) on day 6.

differentiation markers and accumulation of cytoplasmic triglyceride were assessed. Both inhibitors blocked MCE (Fig. 12, which is published as supporting information on the PNAS web site) and drastically curtailed the DNA-binding activity of C/EBPβ as assessed by EMSA (Fig. 7*A*). Specificity of binding was verified by supershifting with antibody to C/EBPβ. In addition, ChIP experiments revealed that both the MAPK and GSK3β inhibitors disrupted binding of C/EBPβ to chromatin associated with the C/EBP regulatory element in the 422/aP2 gene promoter (Fig. 7*B*). Both inhibitors also blocked adipogenesis, as indicated by the inhibition of expression of adipocyte markers, i.e., C/EBPα, PPARγ, and 422/aP2 (Fig. 7*D*), and the accumulation of cytoplasmic triacylglycerol (Fig. 7*C*).

Discussion

C/EBPβ is immediately expressed and phosphorylated by MAPK within 2–4 h after induction of differentiation (Fig. 2 *A* and *B*; see also ref. 10). At this point, however, C/EBPβ lacks DNA-binding activity (Fig. 1*B*; see also ref. 10). After a lag of 12–14 h, C/EBPβ undergoes hyperphosphorylation (Fig. 1*A*) and acquisition of DNA-binding activity as the preadipocytes pass the G_1-S checkpoint. The cells then undergo approximately two rounds of mitosis (MCE) and begin to express genes that produce the adipocyte phenotype (10).

Hyperphosphorylation of C/EBPβ was detected by isoelectric focusing shifts to forms of lower-pI, presumably phosphorylated forms (Fig. 1*A*). The shifts between 4 and 16–24 h after induction of differentiation were accompanied by the acquisition of DNA-binding activity (Fig. 1*B*). This correlation between hyperphosphorylation and binding activity suggested a causal relationship, because phosphatase treatment caused both loss of anionic character and DNA-binding activity by C/EBPβ (Fig. 1). Thus, it appeared that hyperphosphorylation of C/EBPβ, which occurs at the G_1- S checkpoint (10, 29), is responsible for the gain of DNA-binding function.

Here we provide evidence that C/EBPβ is phosphorylated sequentially, first by MAPK and then by GSK3β, at widely separated time points in the differentiation program. Phosphorylation on Thr-188 by MAPK occurs early in the program, i.e., within the first 4 h after induction (Fig. 2*A*), long before C/EBPβ acquires DNA-binding activity. This phosphorylation event is of functional importance, however, because inhibition of MAPK prevents MCE (Fig. 12) and the acquisition of DNA-binding activity (Fig. 7 *A* and *B*), as well as terminal differentiation (Fig. 7 *C* and *D*). Thus, phosphorylation of C/EBPβ by MAPK is required but is insufficient for acquisition of DNA-binding activity.

C/EBPβ is localized within nuclei throughout the differentiation program beginning at 4 h after induction (10), whereas GSK3β is initially localized exclusively in the cytoplasmic compartment. However 12–14 h after induction at the G_1- S checkpoint, $\approx$25% of the GSK3β translocates into the nucleus (29) (Fig. 9), coincident with the onset of hyperphosphorylation and "activation" of C/EBPβ (Fig. 1). Consistent with the role of GSK3β in the hyperphosphorylation of C/EBPβ is the fact that the two newly identified phosphorylation sites in C/EBPβ, i.e., Thr-179 and Ser-184, possess the consensus sequence pattern usually targeted by GSK3 (28).

In vitro phosphorylation of WT and mutant full-length C/EBPβ(LAP) and peptides corresponding to the phosphorylated region of C/EBPβ by MAPK and GSK3β provide definitive evidence that a priming phosphorylation at Thr-188 is required for phosphorylation at Thr-179 and Ser-184 catalyzed by GSK3β (Figs. 4, 5, 8, 10, and 11). Consistent with the predicted phosphorylation pattern for GSK3β substrates (28), phosphorylation of Thr-188 is required for phosphorylation at Thr-179 or Ser-184. MS analysis of the phosphopeptides verified the phosphorylation of phospho-Thr-188 by GSK3β at Thr-179 and Ser-184 (Figs. 8 and 11). Thus, C/EBPβ appears to undergo a "priming" phosphorylation at Thr-188 catalyzed by MAPK shortly after ($\leq$2- to 4-h) induction and subsequently (12–16 h) hyperphosphorylation (presumably by GSK3β) at Thr-179 and Ser-184 (Fig. 2, 4, and 5). Phosphorylation of Thr-179 and Ser-184 correlates closely with the kinetics of translocation of GSK3β from the cytoplasm to the nucleus (29) and the acquisition of DNA-binding activity by C/EBPβ (Figs. 1 and 5).

Although the mechanism by which hyperphosphorylation "activates" DNA-binding activity of C/EBPβ has not been determined, it is likely to involve a conformational change(s) that exposes the binding domain. Johnson's group proposed a model (30) in which a regulatory domain [regulatory domain 2 (RD2)] of C/EBPβ interacts intramolecularly with (and obscures) the DNA-binding domain. Their findings suggested that C/EBPβ exists in a tightly folded conformation in which the DNA-binding domain is masked. It is of interest that the amino acids phosphorylated by MAPK and GSK3β, i.e., Thr-179, Ser-184, and Thr-188, lie within the RD2 regulatory region (residues 162–192) identified by Johnson's group. Thus, our findings are consistent with and lend credence to the suggestion that phosphorylation, in this case hyperphosphorylation of C/EBPβ, may be responsible for the unmasking of the DNA-binding domain of C/EBPβ.

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